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(54) **METHODS FOR TESTING ANTI-THROMBOTIC AGENTS**

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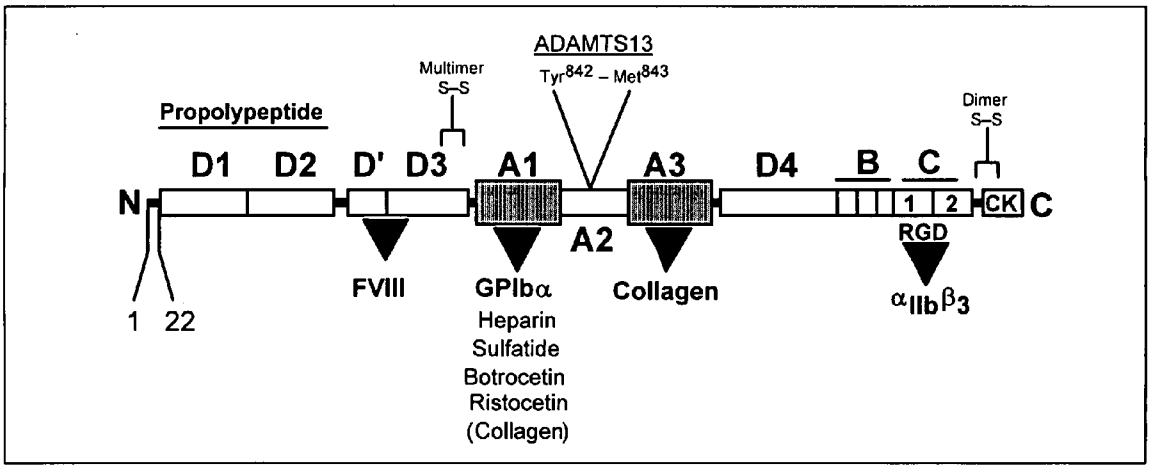
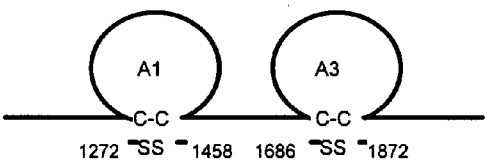
C40B 30/00	(2006.01)
C07K 14/00	(2006.01)
C07H 21/04	(2006.01)
A01K 67/00	(2006.01)

(52) **U.S. Cl.** **424/1.11**; 424/9.1; 424/9.3; 435/29; 435/320.1; 506/7; 530/350; 536/23.1; 800/13; 800/14; 800/18

(57) **ABSTRACT**

The invention provides a transgenic non-human animal expressing von Willebrand Factor A1 protein containing at least one mutation selected from the group consisting of: 1263P>S, 1269N>D, 1274K>R, 1287M>R, 1302G>D, 1308H>R, 1313R>W, 1314I>V, 1326R>H, 1329L>I, 1330E>G, 1333A>D, 1344T>A, 1347I>V, 1350T>A, 1370G>S, 1379H>R, 1381T>A, 1385T>M 1391P>Q, 1394A>S, 1397L>F, 1421S>N, 1439L>V, 1442G>S, 1449R>Q, 1466A>P, 1469Q>L, 1472Q>H, 1473V>M, 1475H>Q, 1479S>G, and any combination thereof.





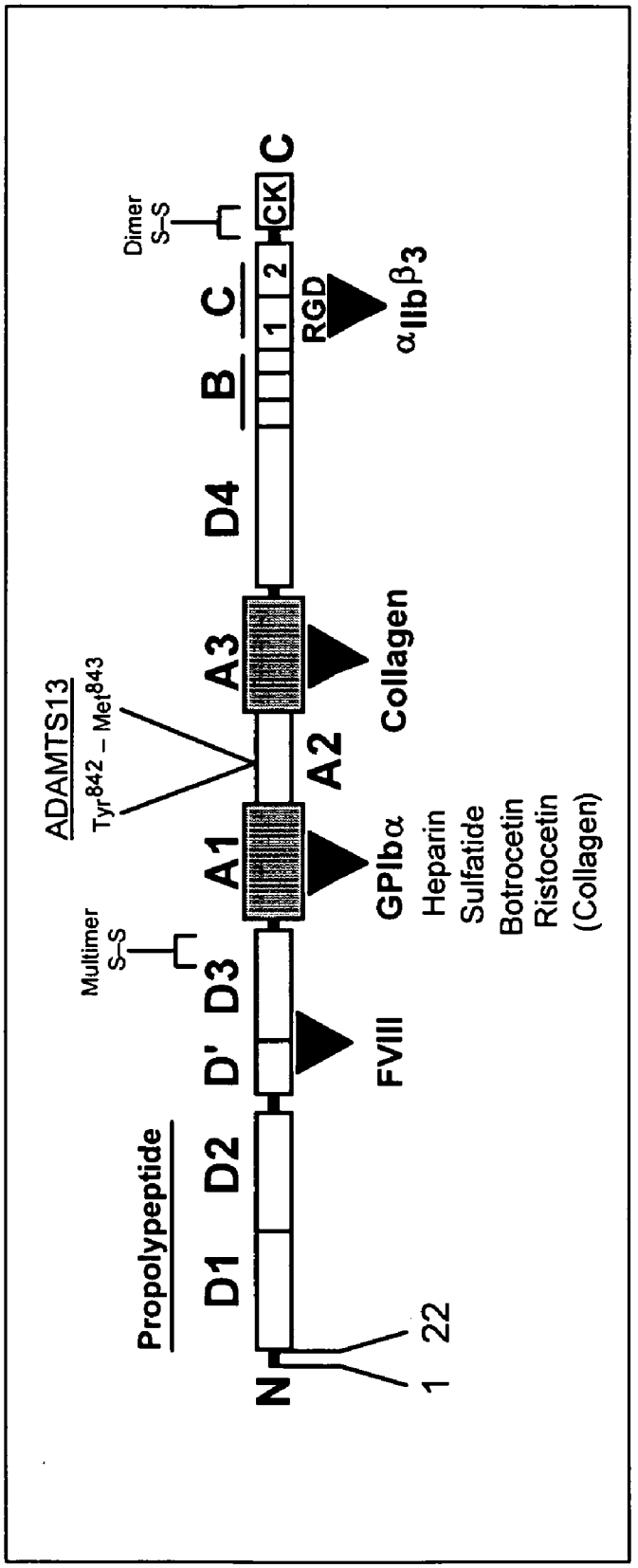

Propolypeptide
D1
D2
D'
D3
A1
A2
A3
D4
B
C
CK
N
1
22
Multimer S-S
Dimer S-S
ADAMTS13
Tyr842 – Met843
FVIII
GPIbα
Heparin
Sulfatide
Botrocetin
Ristocetin
(Collagen)
Collagen
RGD
αIIbβ3
A1
A3
C-C
C-C
1272 SS 1458
1686 SS 1872

FIG. 1A

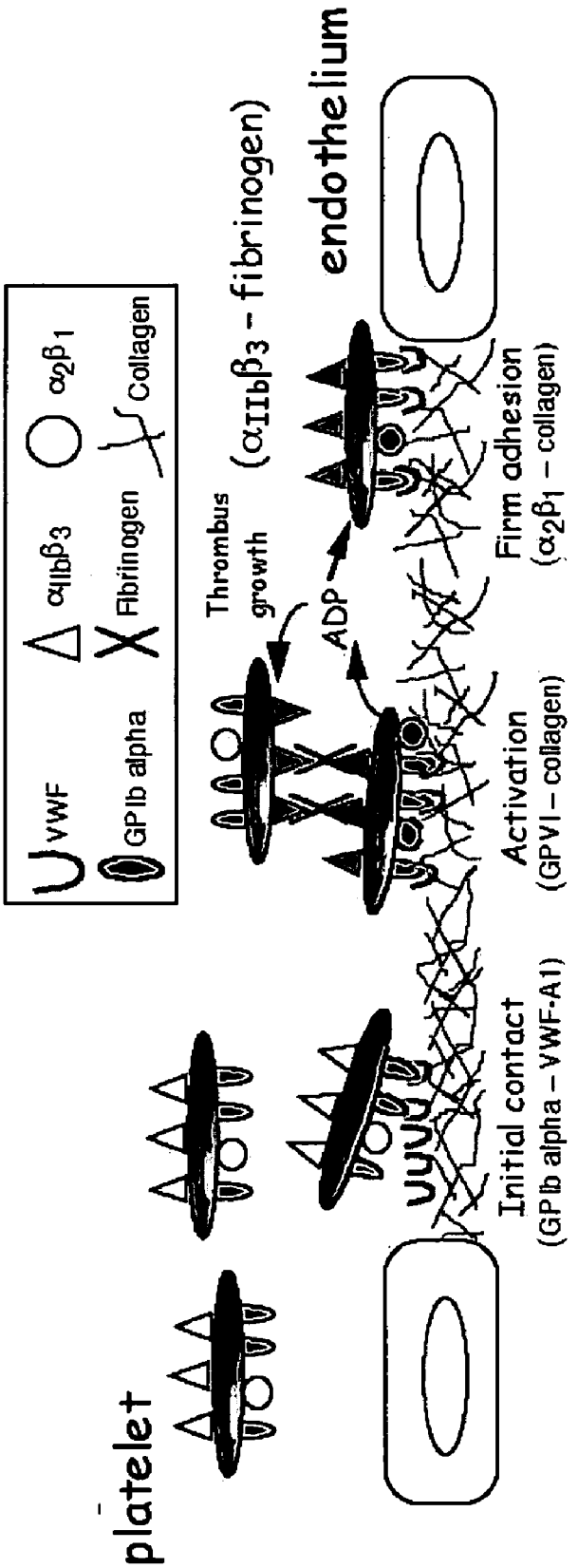
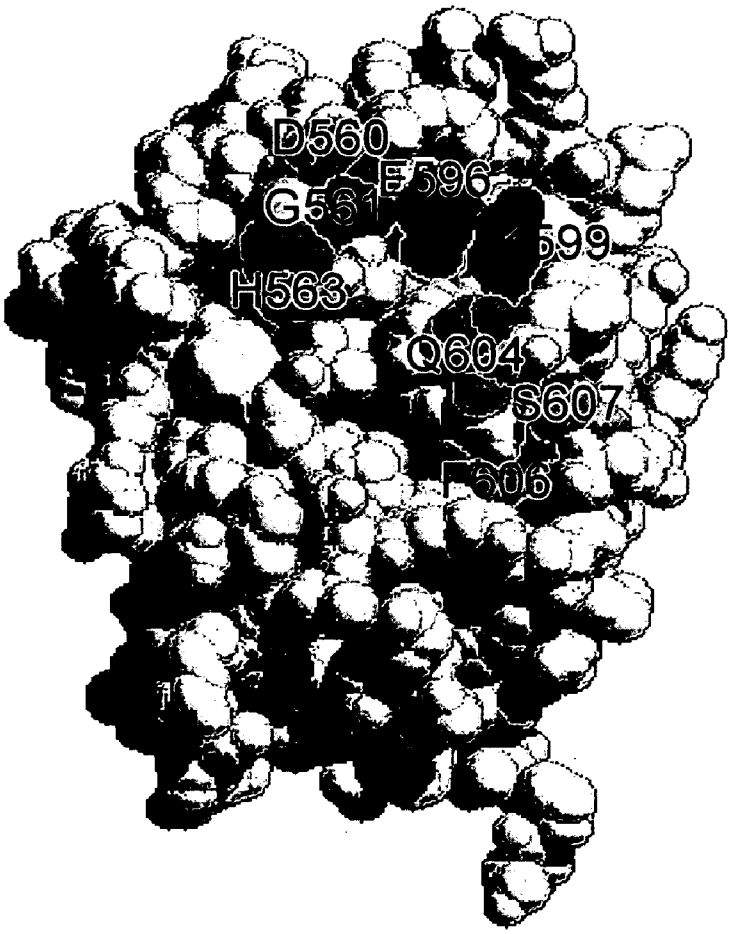

VWF
GPIb alpha
$\alpha_{IIb}\beta_3$
Fibrinogen
$\alpha_2\beta_1$
Collagen
platelet
Initial contact
(GPIb alpha – VWF-A1)
Activation
(GPVI – collagen)
ADP
Thrombus
growth
($\alpha_{IIb}\beta_3$ - fibrinogen)
Firm adhesion
($\alpha_2\beta_1$ – collagen)
endothelium

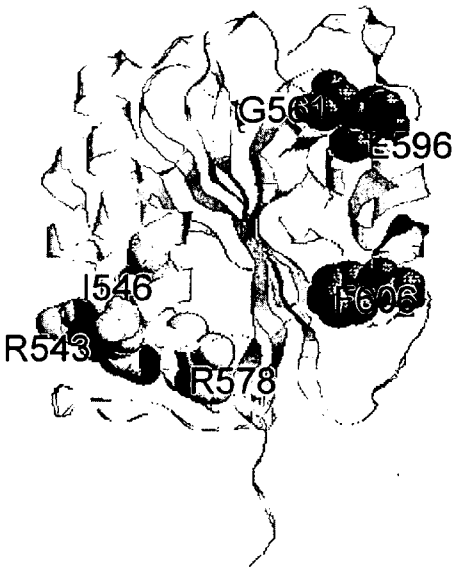
FIG. 1B



* standard vWF nomenclature	aa designation in image
D1323	D560
G1324	G561
H1326	H563
E1359	E596
K1362	K599
Q1367	Q604
F1369	F606
S1370	S607

FIG. 2

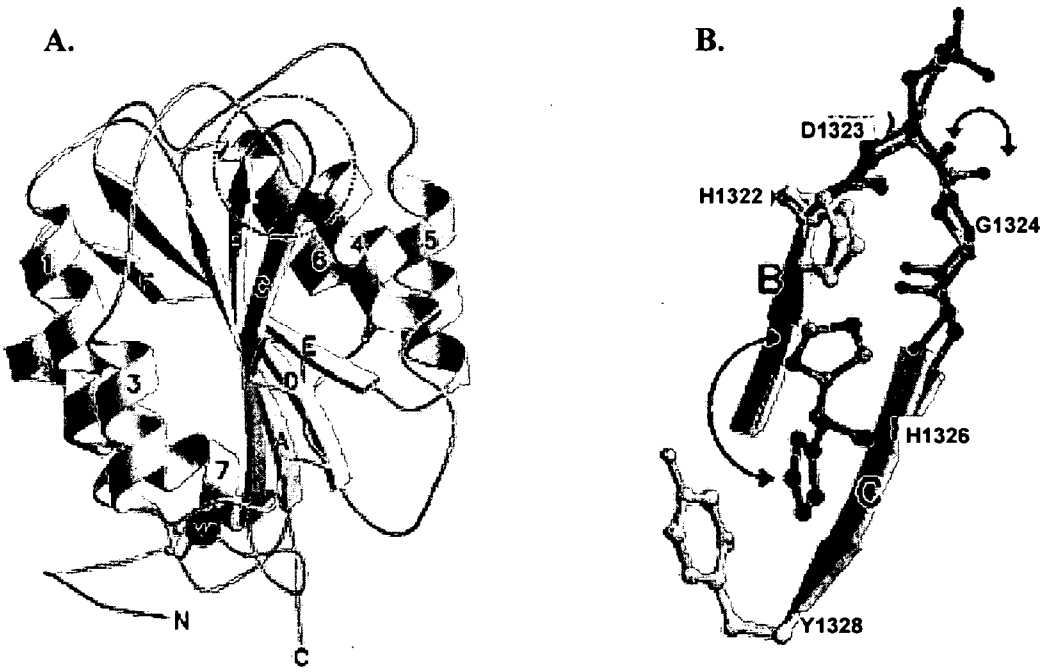
A.



B.

* standard vWF nomenclature	aa designation in image
R1306	R543
I1309	I546
G1324	G561
R1341	R578
E1359	E596
F1369	F606

FIG. 3



* standard vWF nomenclature	aa designation in image
I1309	I546

FIG. 4

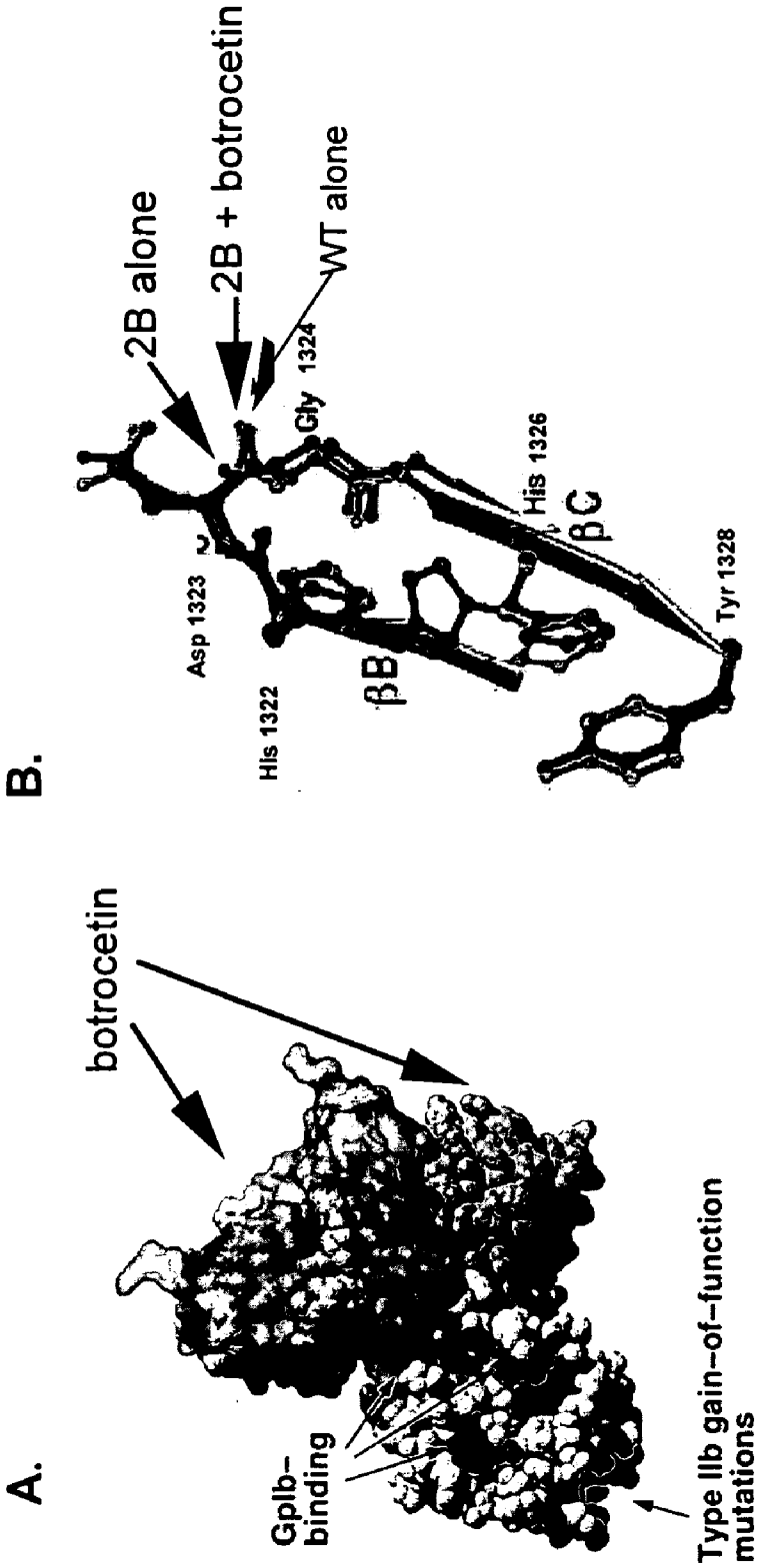

A.
botrocetin
GpIb-binding
Type IIb gain-of-function mutations
B.
2B alone
2B + botrocetin
WT alone
Gly 1324
His 1326
βC
Asp 1323
His 1322
βB
Tyr 1328

FIG. 5

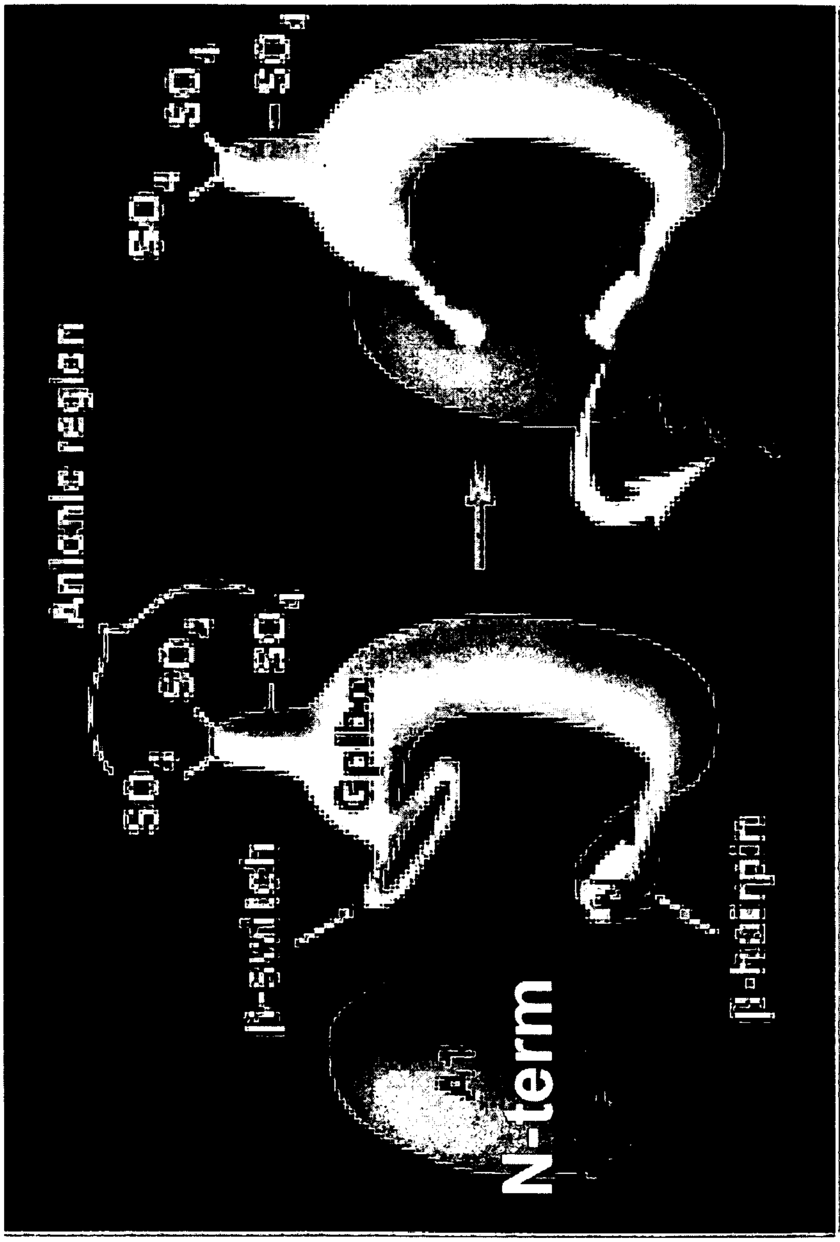
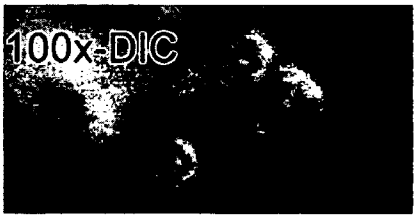

Anionic region
SO4
SO4
SO4
SO4
SO4
SO4
GpIbα
β-switch
β-hairpin
N-term

FIG. 6

A.



B.

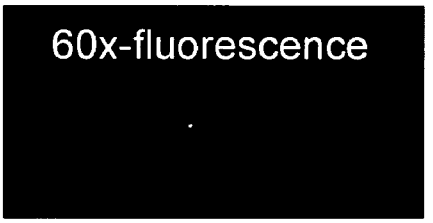


FIG. 7

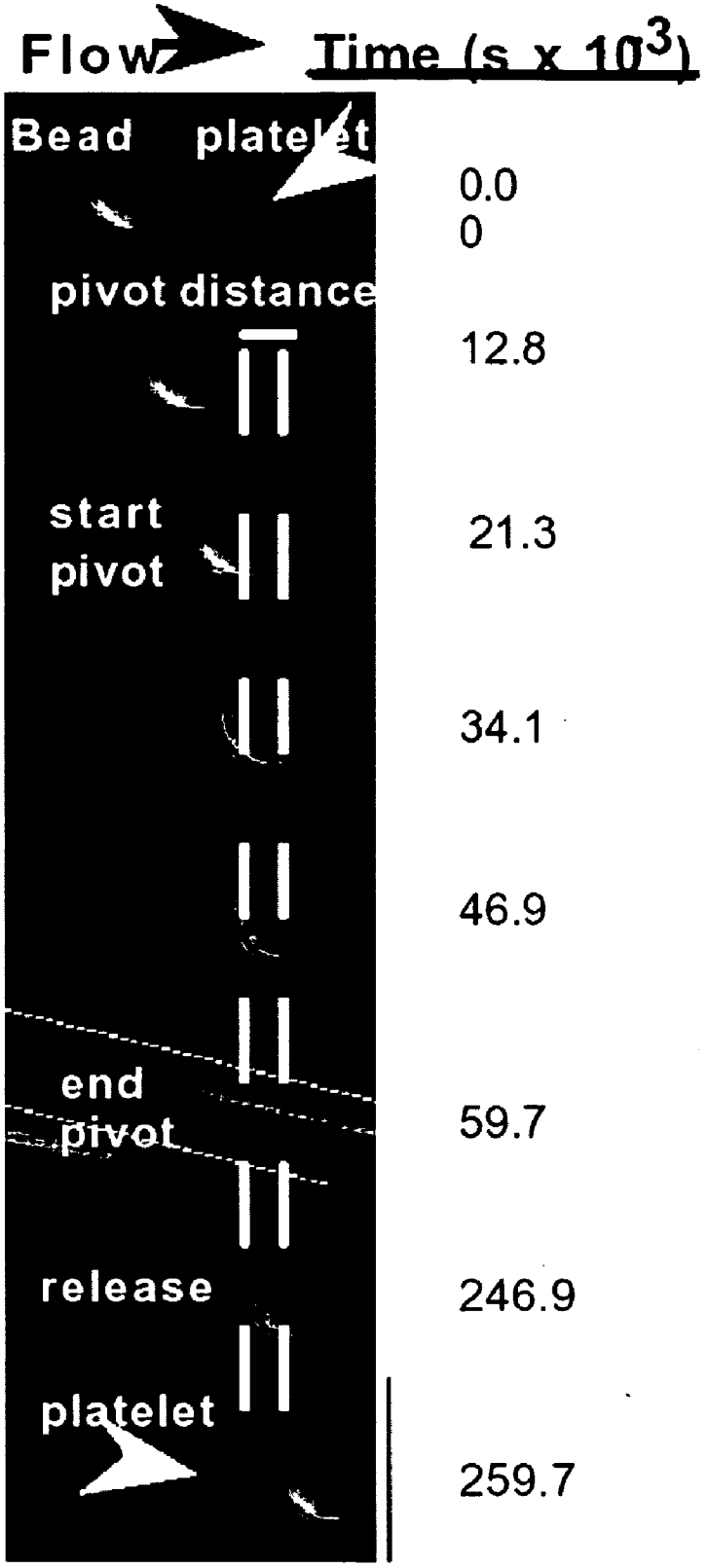

Flow
Time (s x 10^3)
Bead
platelet
0.0
0
pivot distance
12.8
start
pivot
21.3
34.1
46.9
end
pivot
59.7
release
246.9
platelet
259.7

FIG. 8A

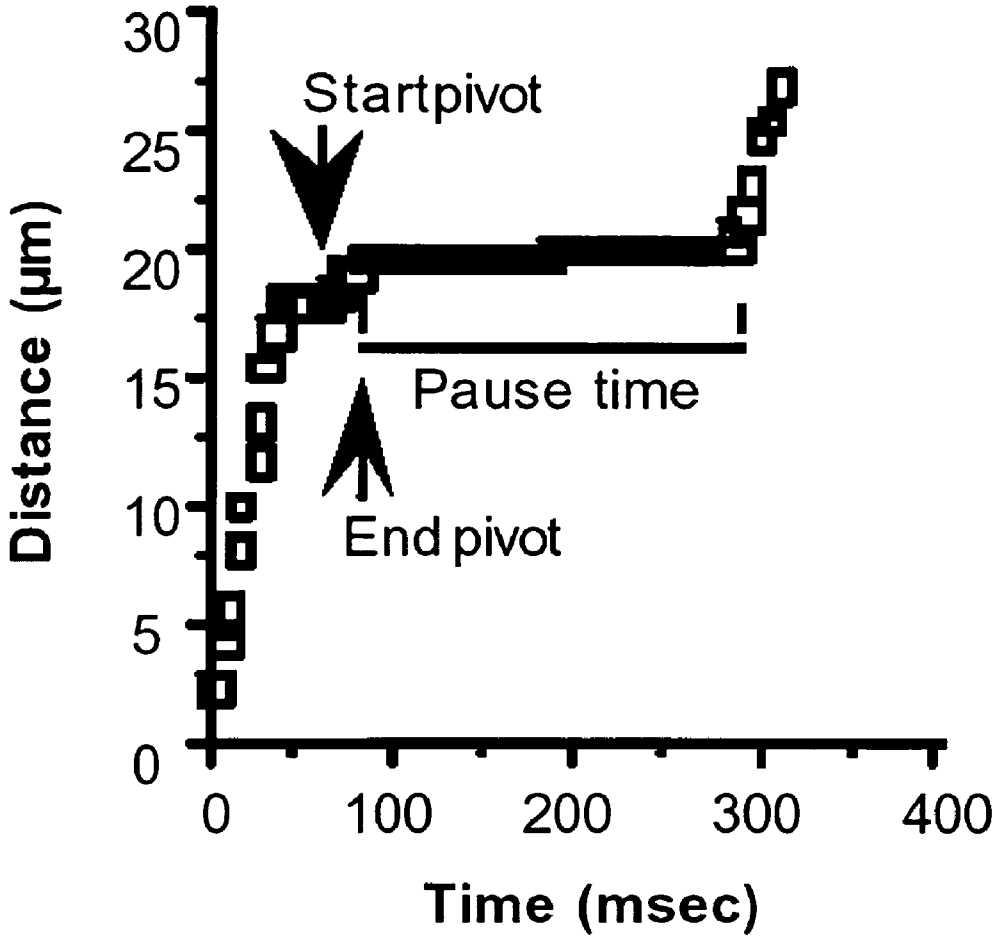
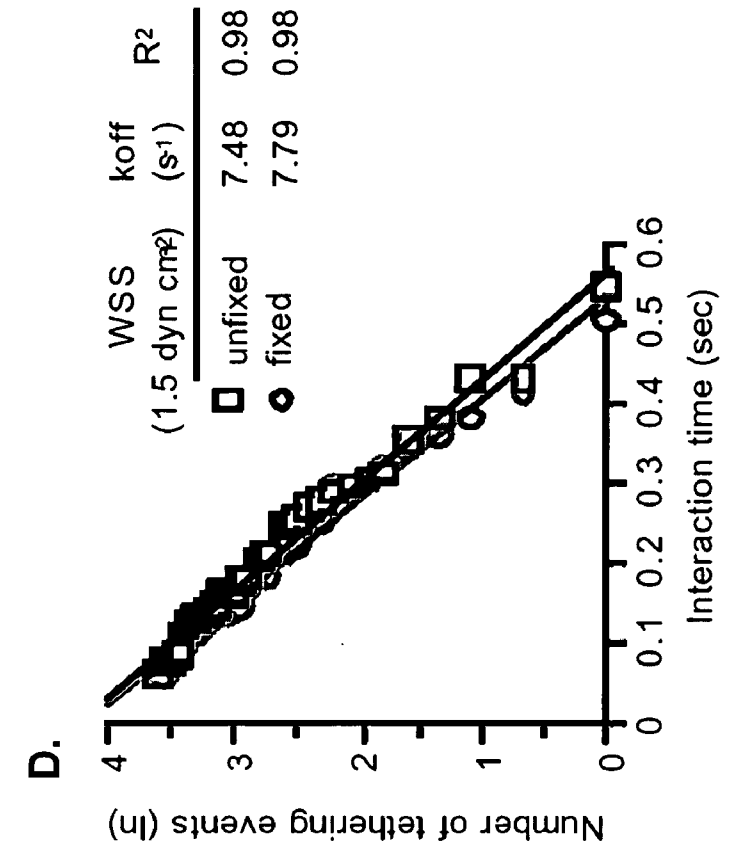

30
25
20
15
10
5
0
Distance (μm)
Startpivot
Pause time
End pivot
0
100
200
300
400
Time (msec)

FIG. 8B


D.
WSS (1.5 dyn cm²)
koff (s⁻¹)
R²
unfixed 7.48 0.98
fixed 7.79 0.98
Number of tethering events (ln)
Interaction time (sec)
0 0.1 0.2 0.3 0.4 0.5 0.6
0 1 2 3 4

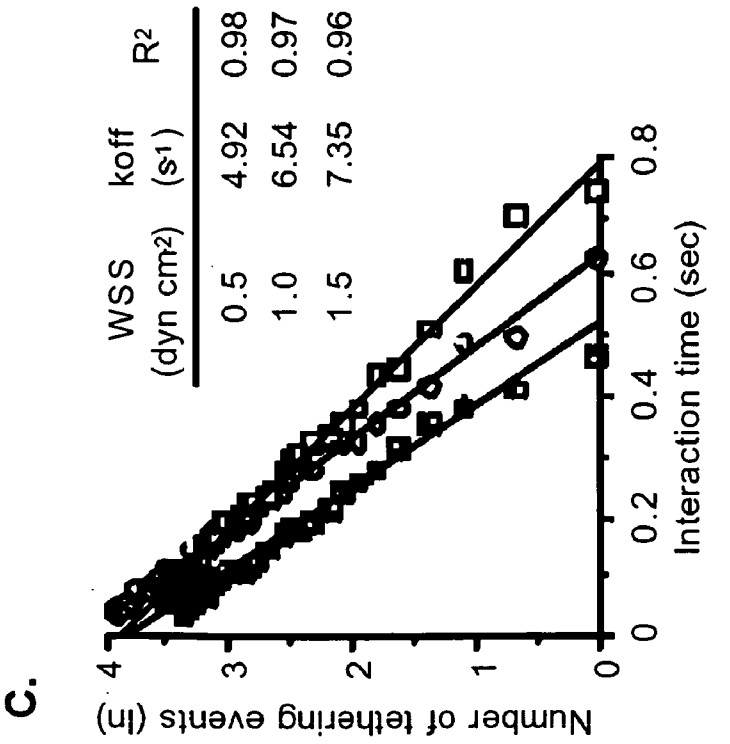

C.
WSS (dyn cm²)
koff (s⁻¹)
R²
0.5 4.92 0.98
1.0 6.54 0.97
1.5 7.35 0.96
Number of tethering events (ln)
Interaction time (sec)
0 0.2 0.4 0.6 0.8
0 1 2 3 4

FIG. 8C-D

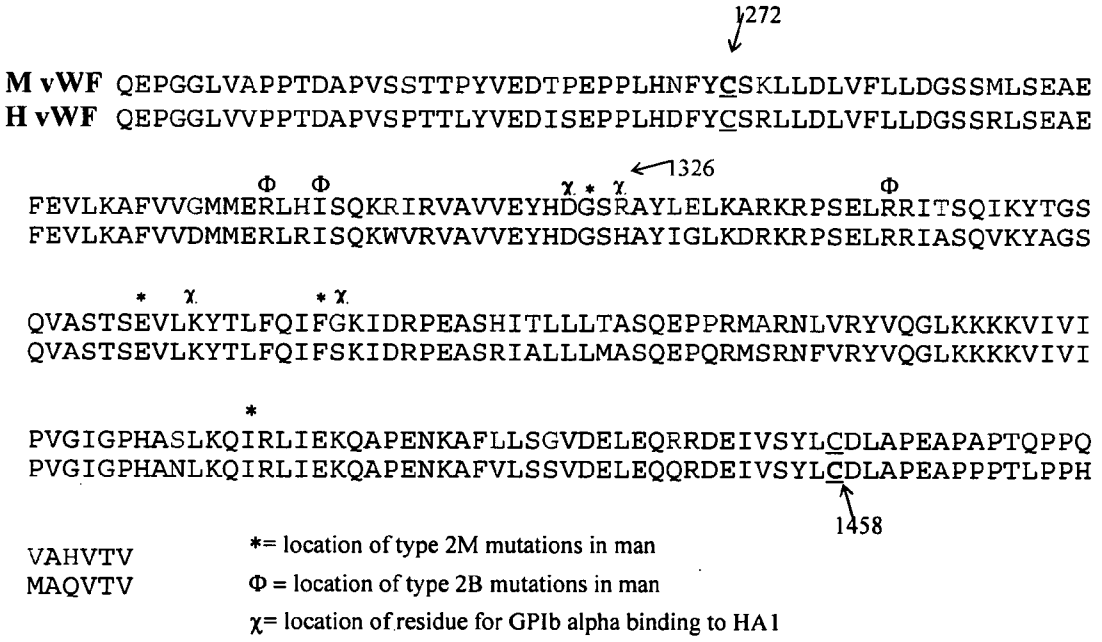

1272
M vWF QEPGGLVAPPTDAPVSSTTPYVEDTPEPPLHNFYCSKLLDLVFLLDGSSMLSEAE
H vWF QEPGGLVVPPTDAPVSPTTLYVEDISEPPLHDFYCSRLLDLVFLLDGSSRLSEAE
1326
Φ Φ χ * χ Φ
FEVLKAFVVGMMERLHISQKRIRVAVVEYHDGSRAYLELKARKRPSELRRITSQIKYTGS
FEVLKAFVVDMMERLRISQKWVRVAVVEYHDGSHAYIGLKDRKRPSELRRIASQVKYAGS
* χ * χ
QVASTSEVLKYTLFQIFGKIDRPEASHITLLLTASQEPPRMARNLVRYVQGLKKKKVIVI
QVASTSEVLKYTLFQIFSKIDRPEASRIALLLMASQEPQRMSRNFVRYVQGLKKKKVIVI
*
PVGIGPHASLKQIRLIEKQAPENKAFLLSGVDELEQRRDEIVSYLCDLAPEAPAPTQPPQ
PVGIGPHANLKQIRLIEKQAPENKAFVLSSVDELEQQRDEIVSYLCDLAPEAPPPTLPPH
1458
VAHVTV
MAQVTV
*= location of type 2M mutations in man
Φ = location of type 2B mutations in man
χ= location of residue for GPIb alpha binding to HA1

FIG. 9

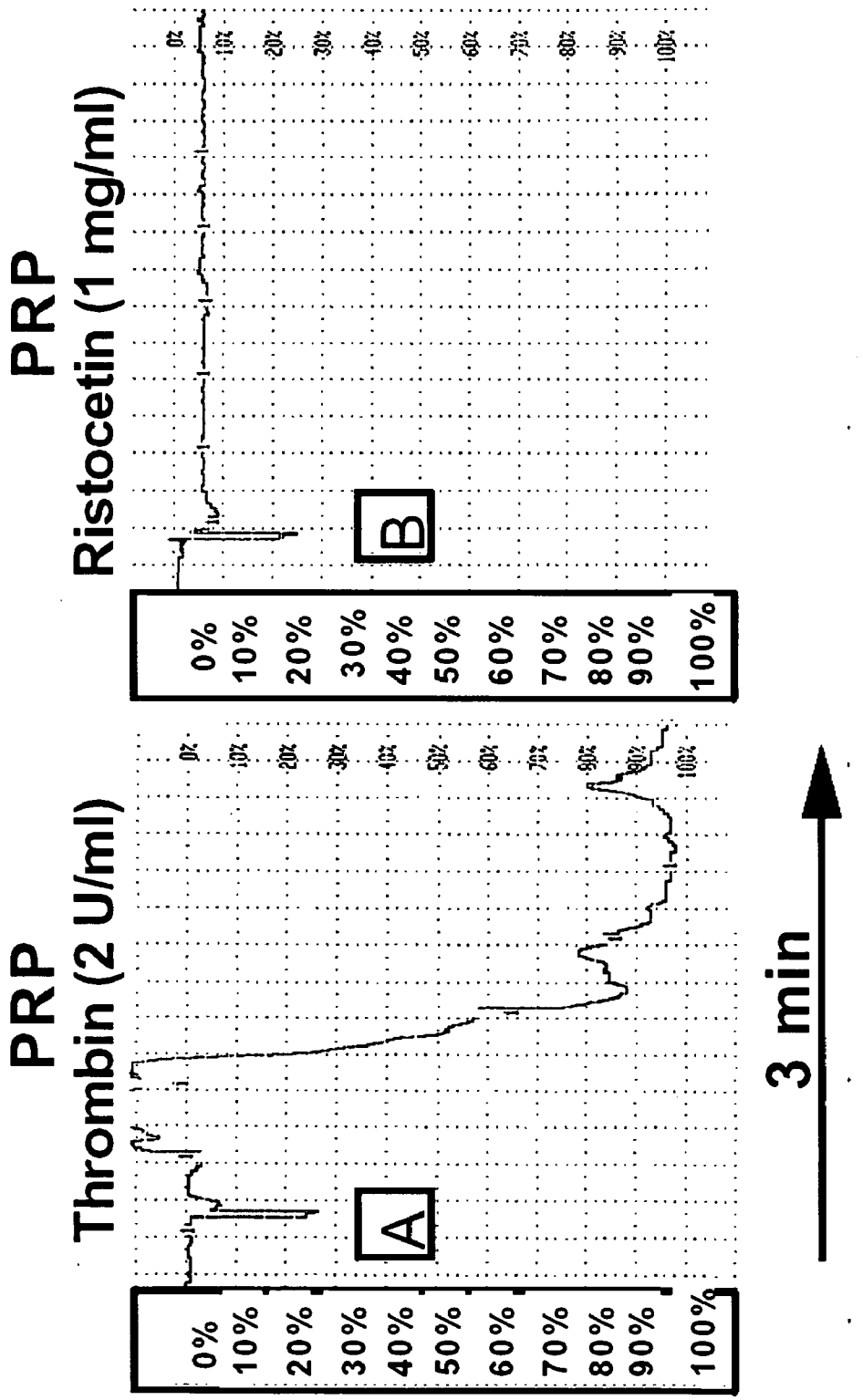

PRP
Thrombin (2 U/ml)
PRP
Ristocetin (1 mg/ml)
A
B
0%
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%
3 min

FIG. 10A-B

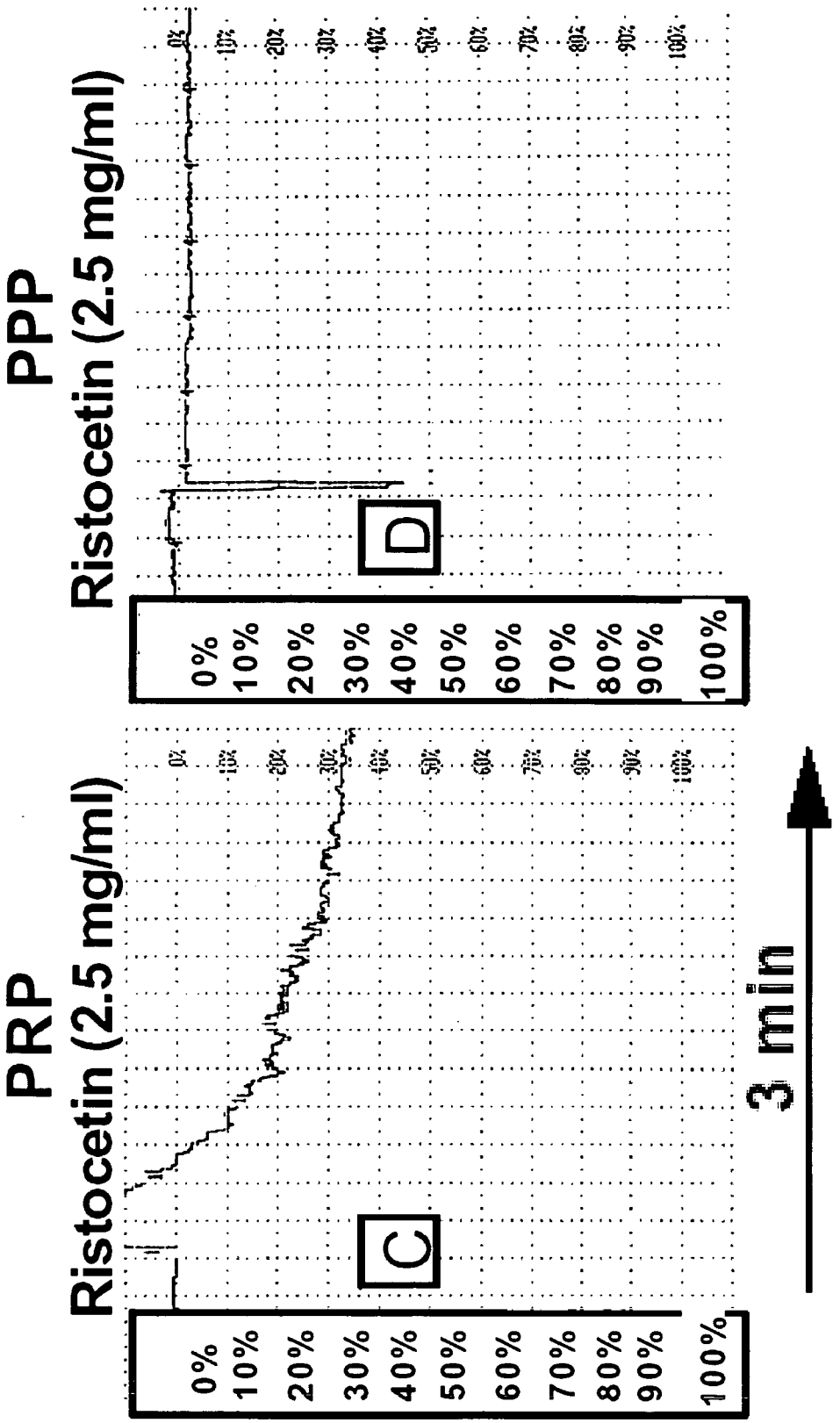

PRP
Ristocetin (2.5 mg/ml)
C
0%
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%
3 min
PPP
Ristocetin (2.5 mg/ml)
D
0%
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%

FIG. 10C-D

A.


hvWF
mvWF
Lane: 1
2

B.

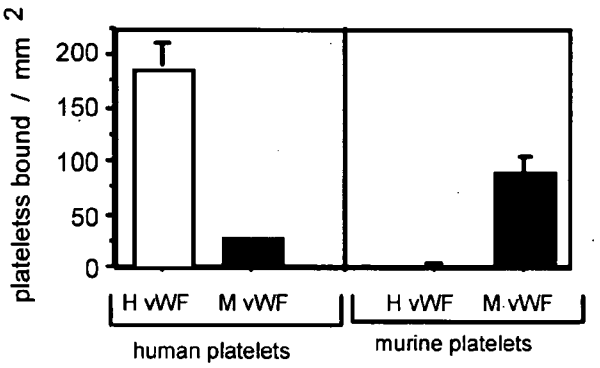

plateletss bound / mm 2
200
150
100
50
0
H vWF
M vWF
H vWF
M vWF
human platelets
murine platelets

FIG. 11

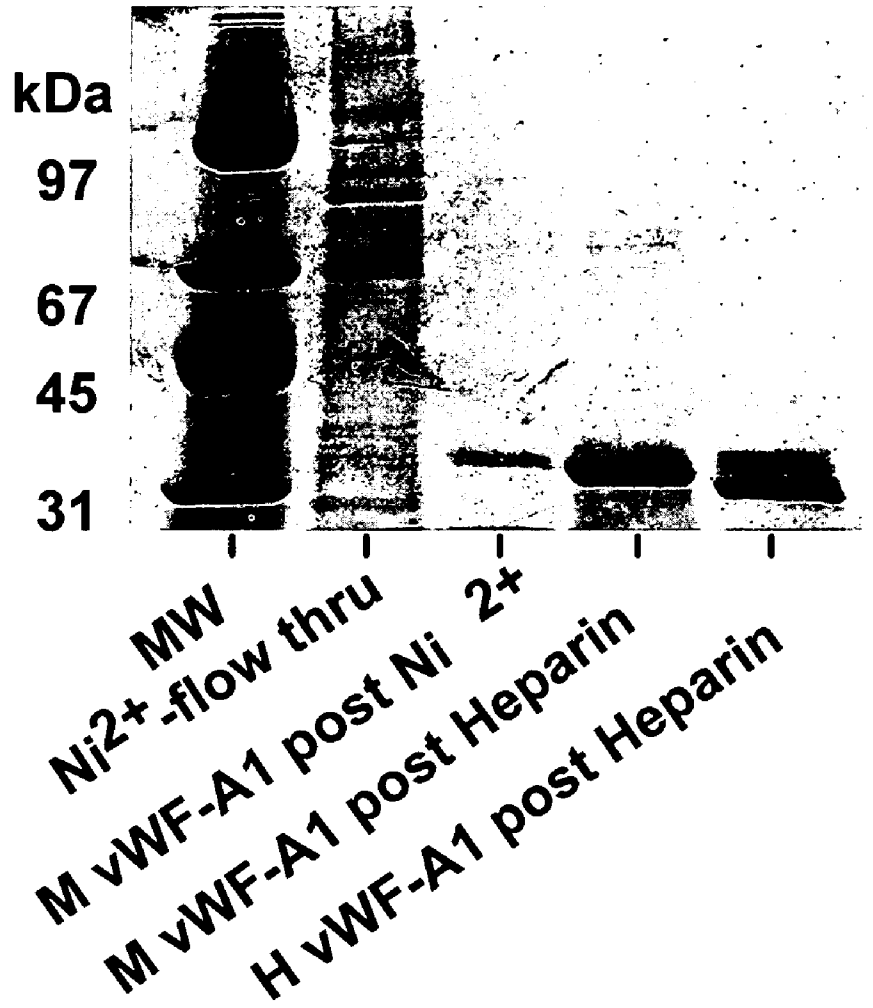
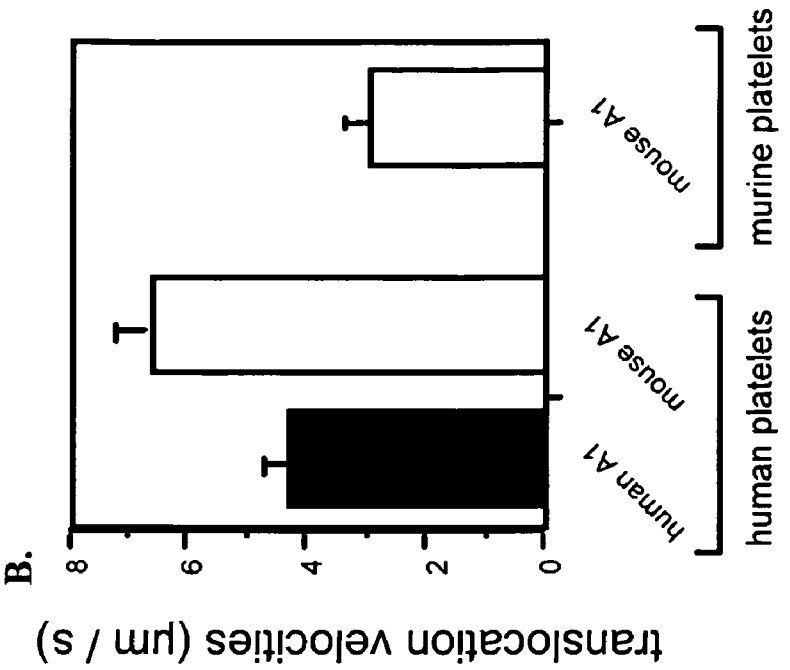

kDa
97
67
45
31
MW
Ni2+ -flow thru
M vWF-A1 post Ni 2+
M vWF-A1 post Heparin
H vWF-A1 post Heparin

FIG. 12


B.
translocation velocities (µm / s)
0
2
4
6
8
human A1
mouse A1
mouse A1
human platelets
murine platelets

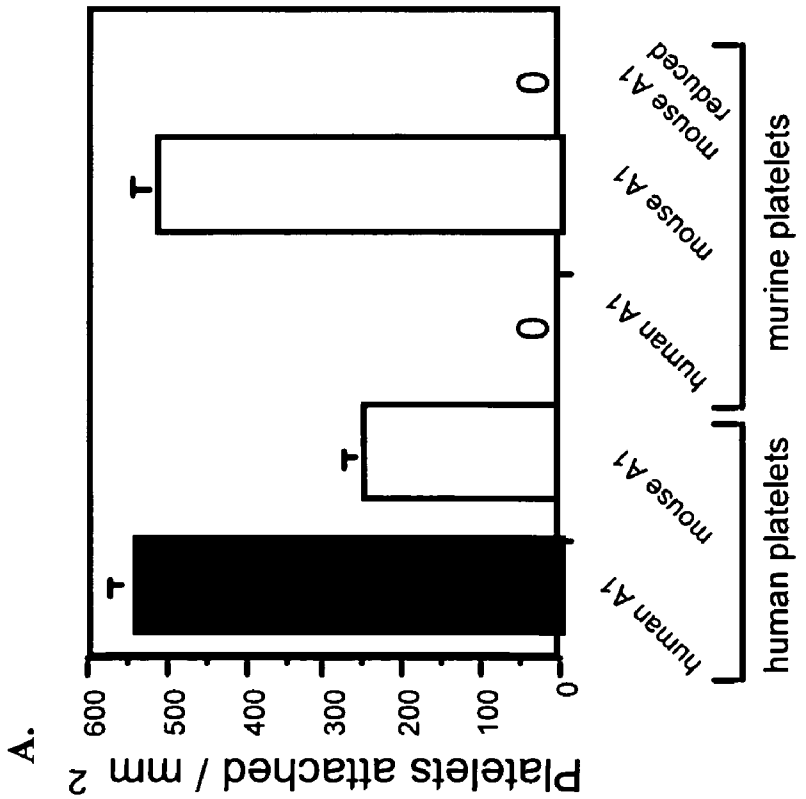
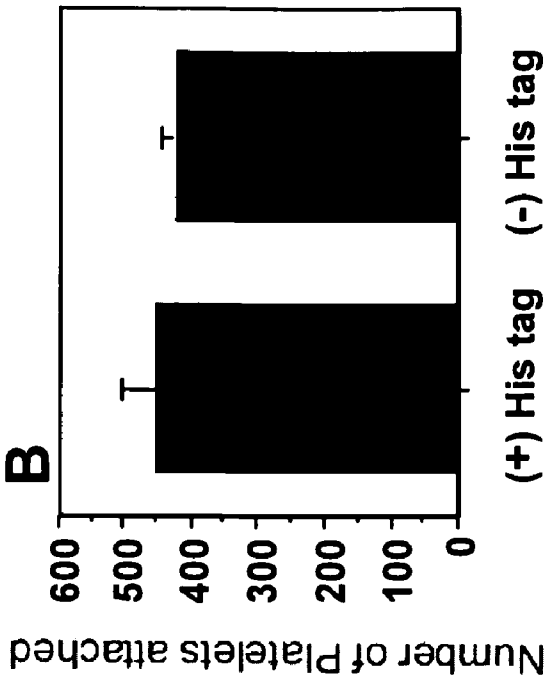

A.
Platelets attached / mm 2
0
100
200
300
400
500
600
human A1
mouse A1
human A1
mouse A1
mouse A1 reduced
human platelets
murine platelets

FIG. 13


B
Number of Platelets attached
600
500
400
300
200
100
0
(+) His tag
(-) His tag

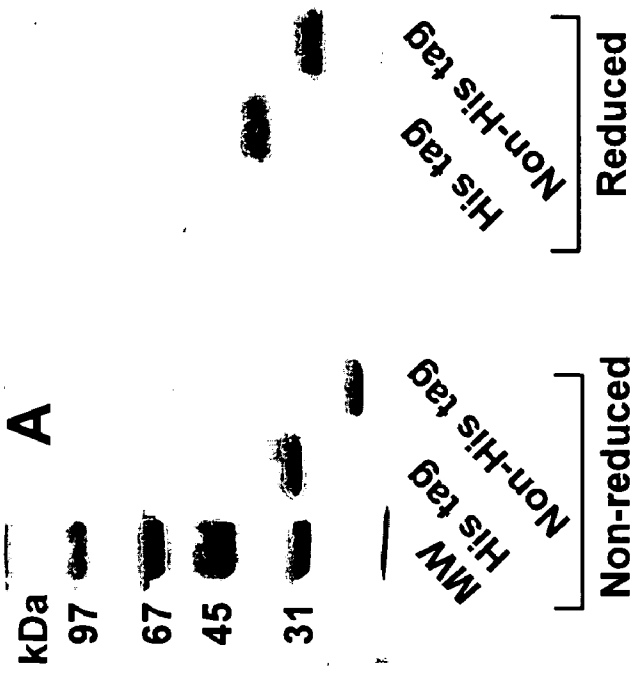

A
kDa
97
67
45
31
MW
His tag
Non-His tag
Non-reduced
His tag
Non-His tag
Reduced

FIG. 14

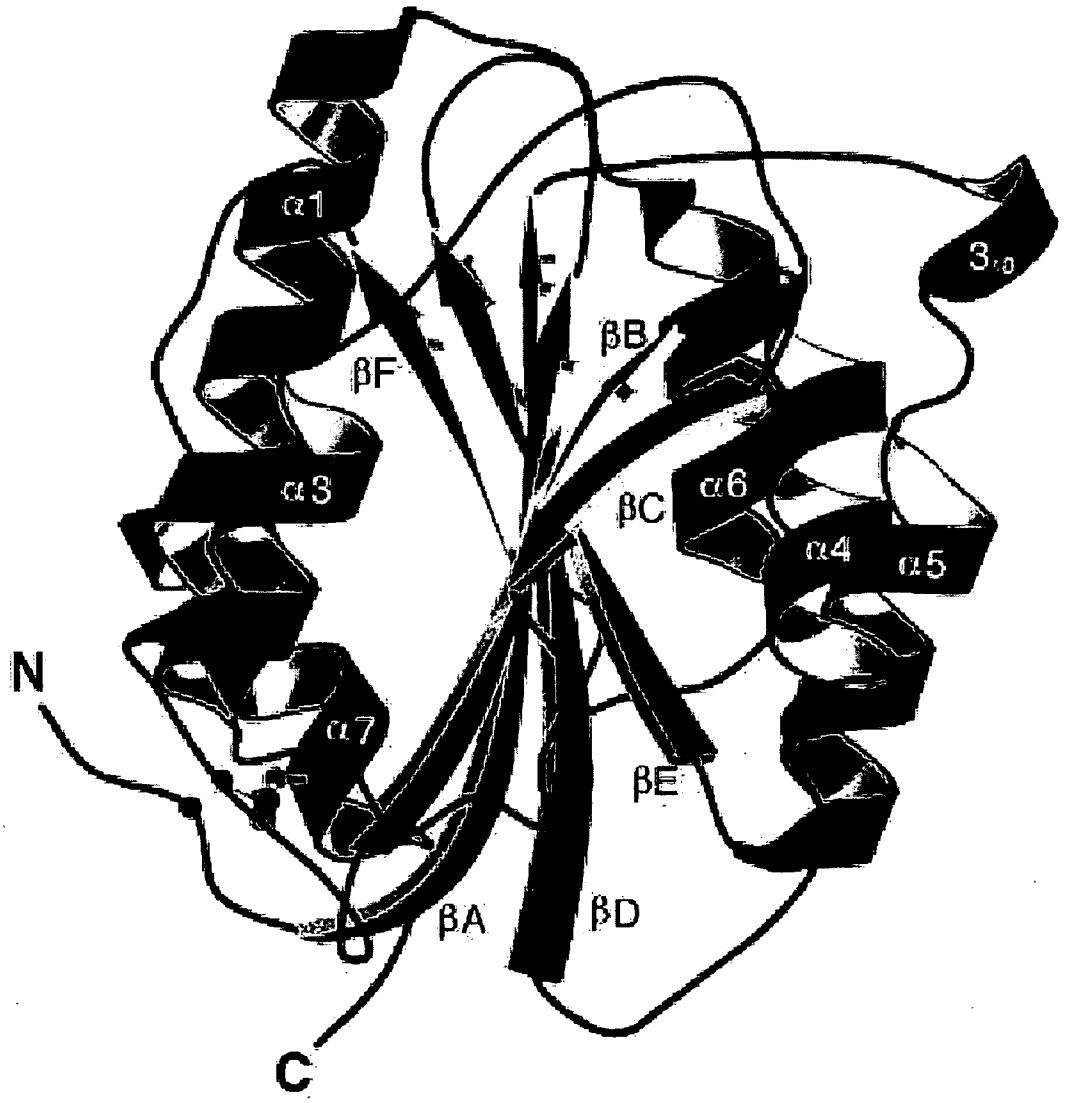


FIG. 15A

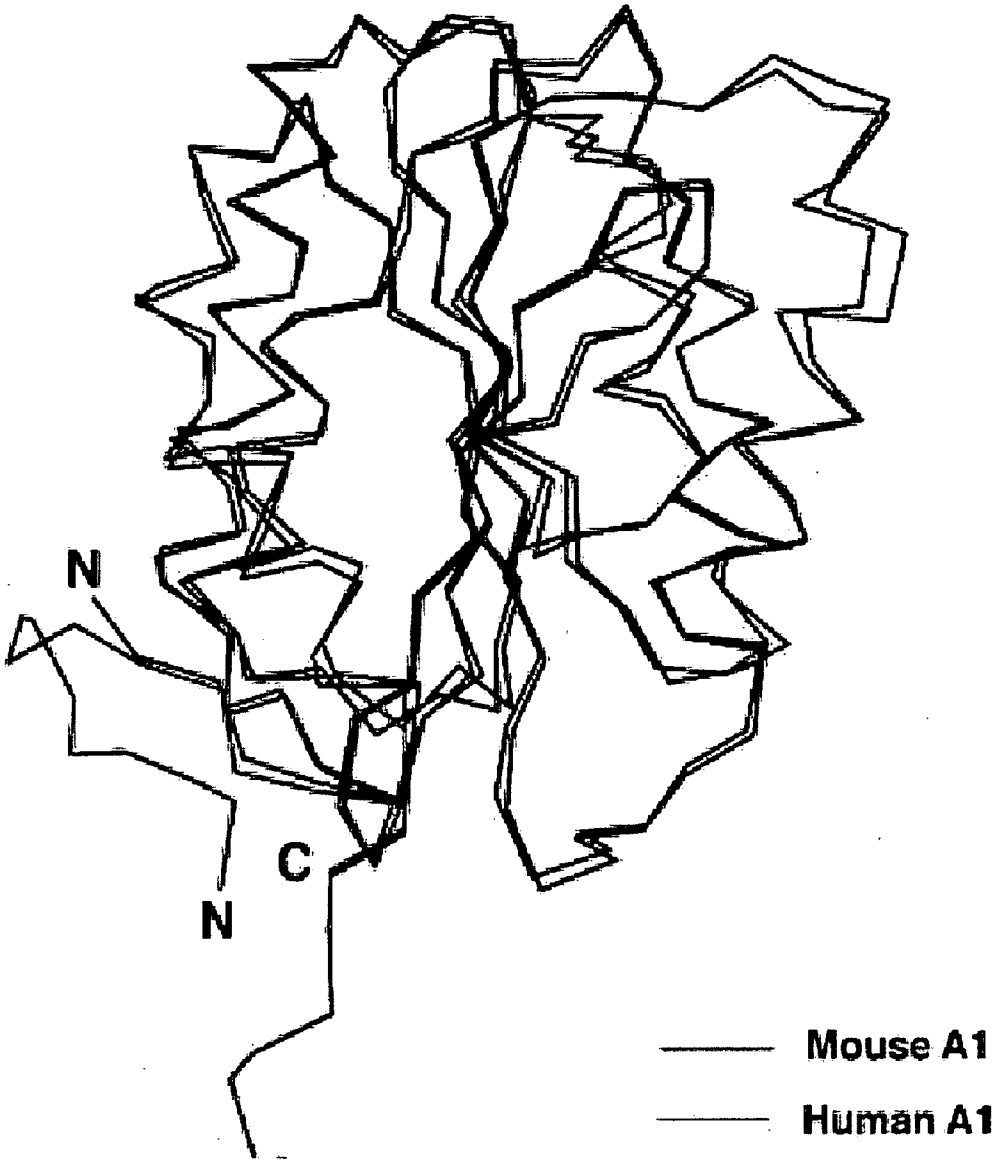


FIG. 15B

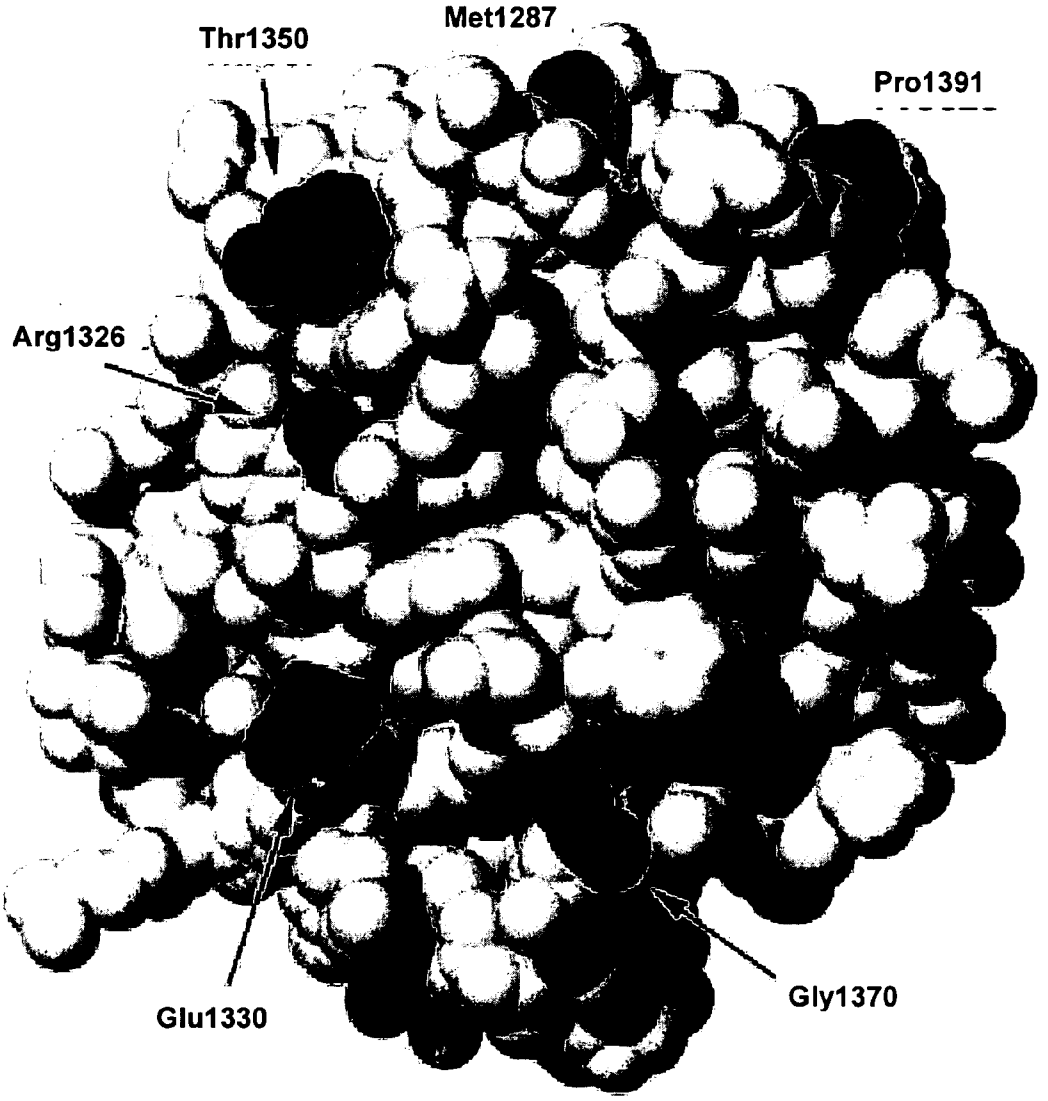
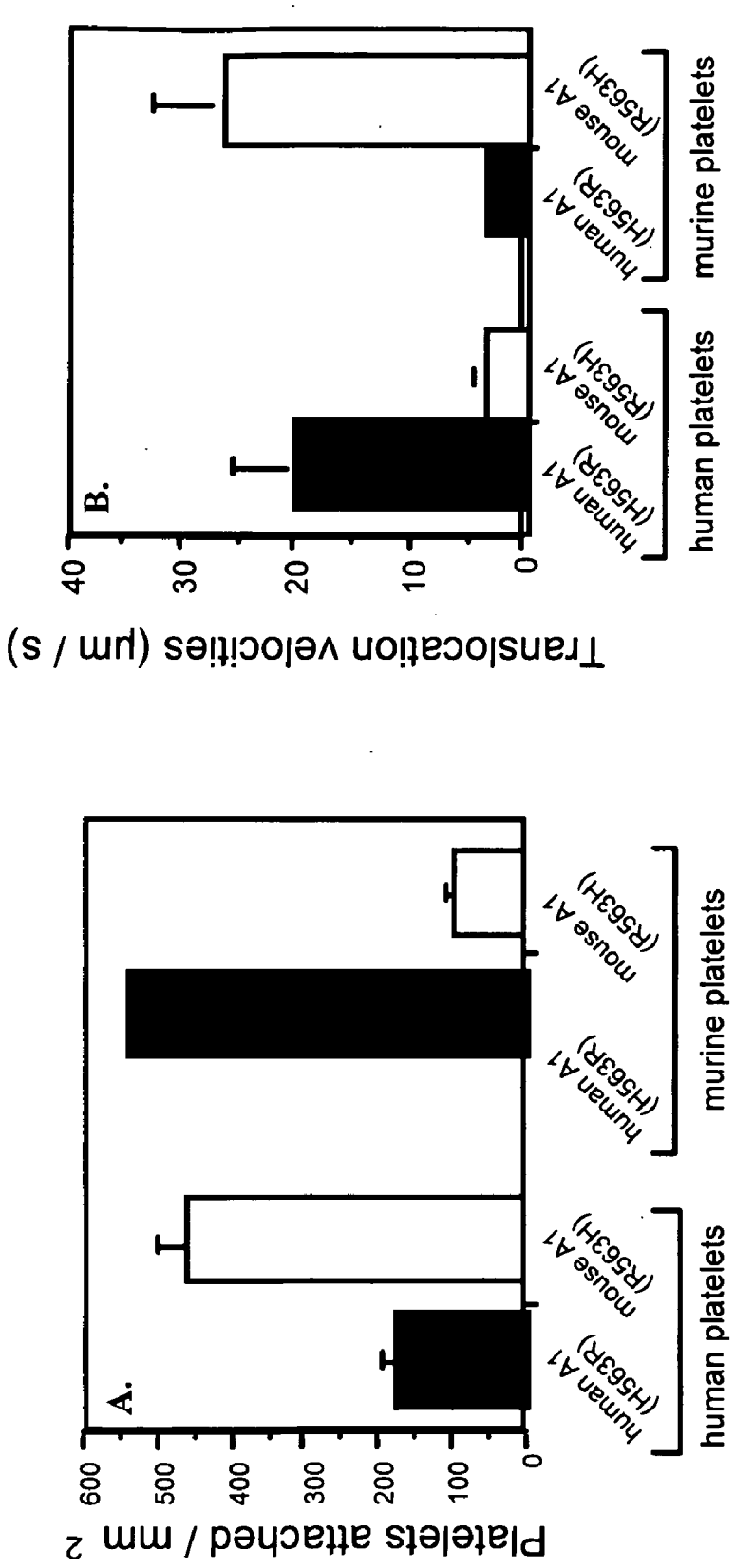


FIG. 15C



*Using standard nomenclature for vWF, the H563R mutation corresponds to H1326R and the R563H mutation corresponds to R1326H.

FIG. 16

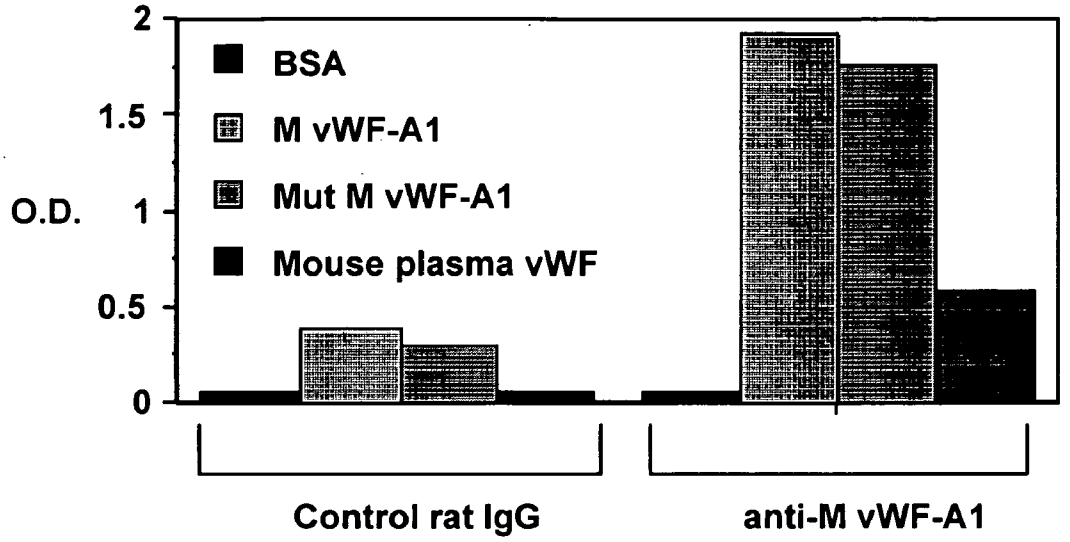

2
1.5
1
0.5
0
O.D.
BSA
M vWF-A1
Mut M vWF-A1
Mouse plasma vWF
Control rat IgG
anti-M vWF-A1

FIG. 17

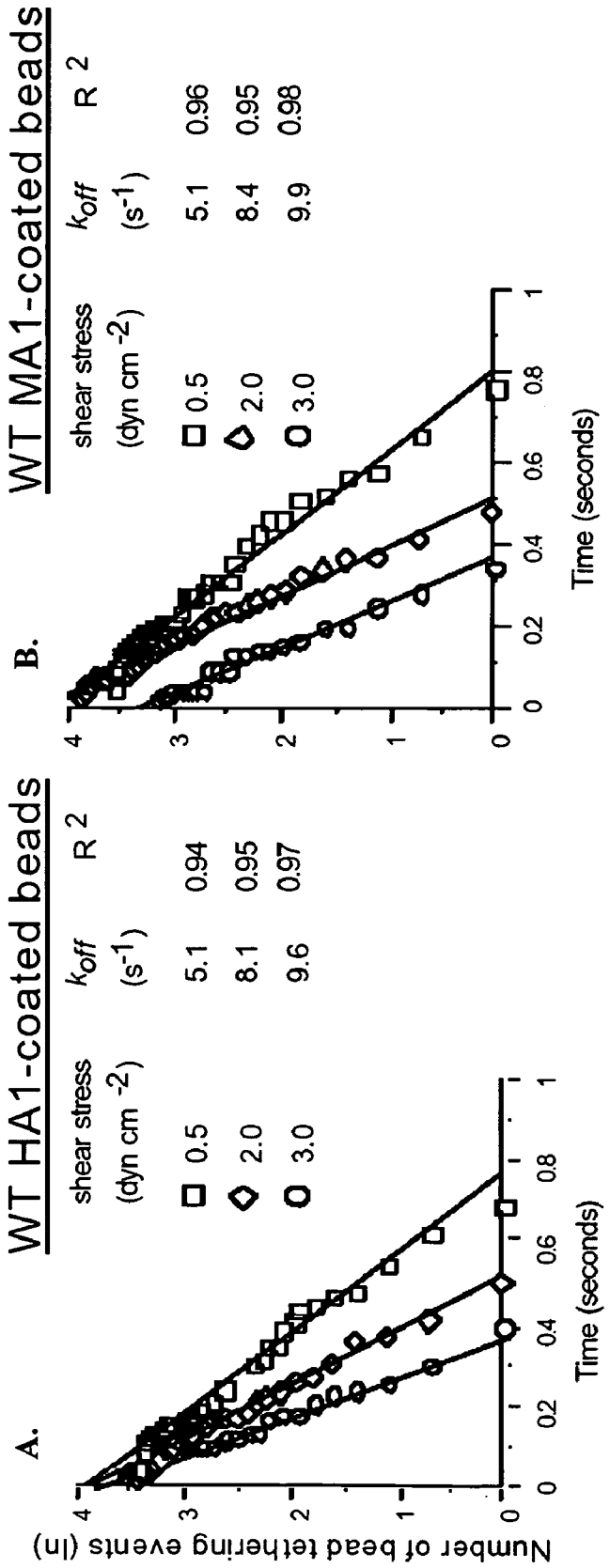

A.
WT HA1-coated beads
shear stress (dyn cm -2)
k_{off} (s^{-1})
R 2
0.5 5.1 0.94
2.0 8.1 0.95
3.0 9.6 0.97
Number of bead tethering events (ln)
Time (seconds)
B.
WT MA1-coated beads
shear stress (dyn cm -2)
k_{off} (s^{-1})
R 2
0.5 5.1 0.96
2.0 8.4 0.95
3.0 9.9 0.98
Time (seconds)

FIG. 18A-B

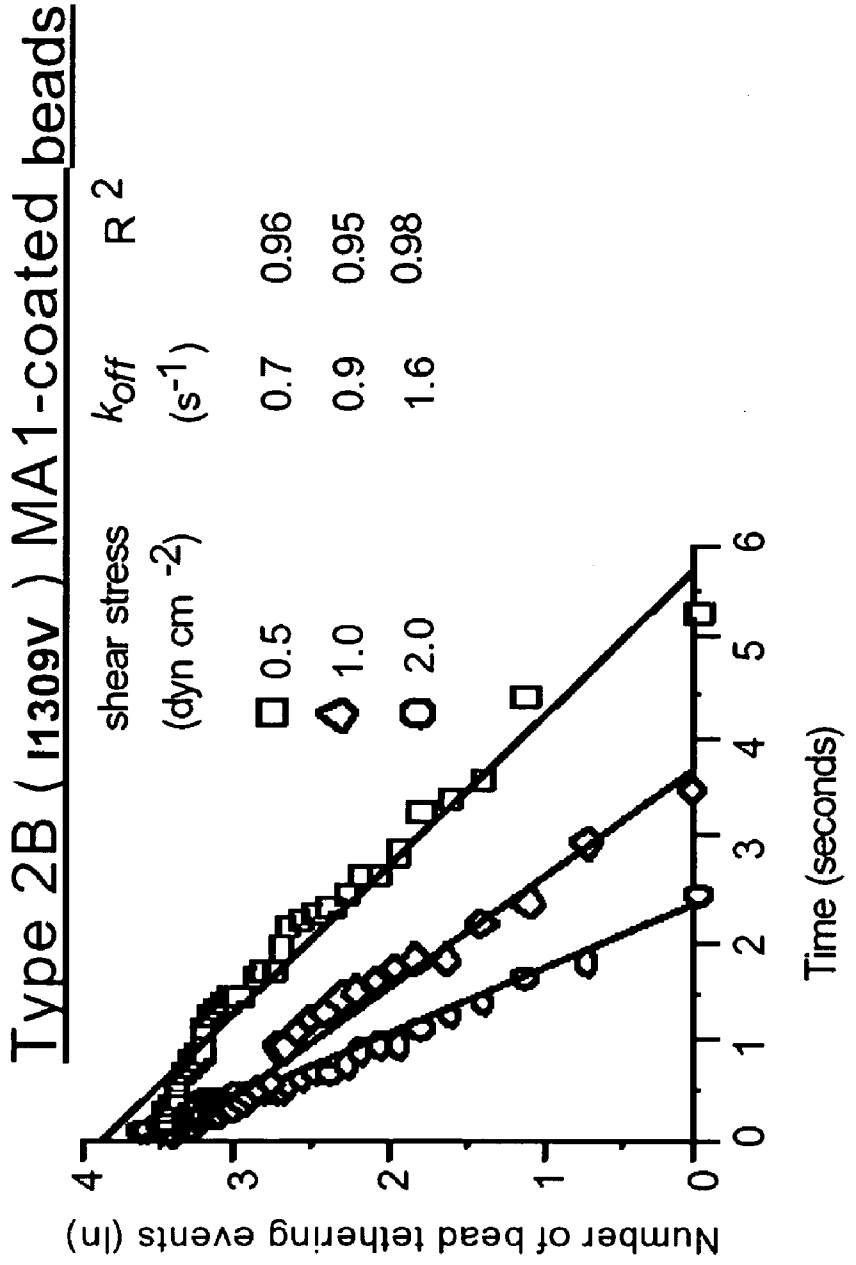

Type 2B (I1309V) MA1-coated beads
shear stress (dyn cm -2)
koff (s-1)
R 2
0.5
0.7
0.96
1.0
0.9
0.95
2.0
1.6
0.98
Number of bead tethering events (ln)
Time (seconds)
0
1
2
3
4
5
6

FIG. 18C

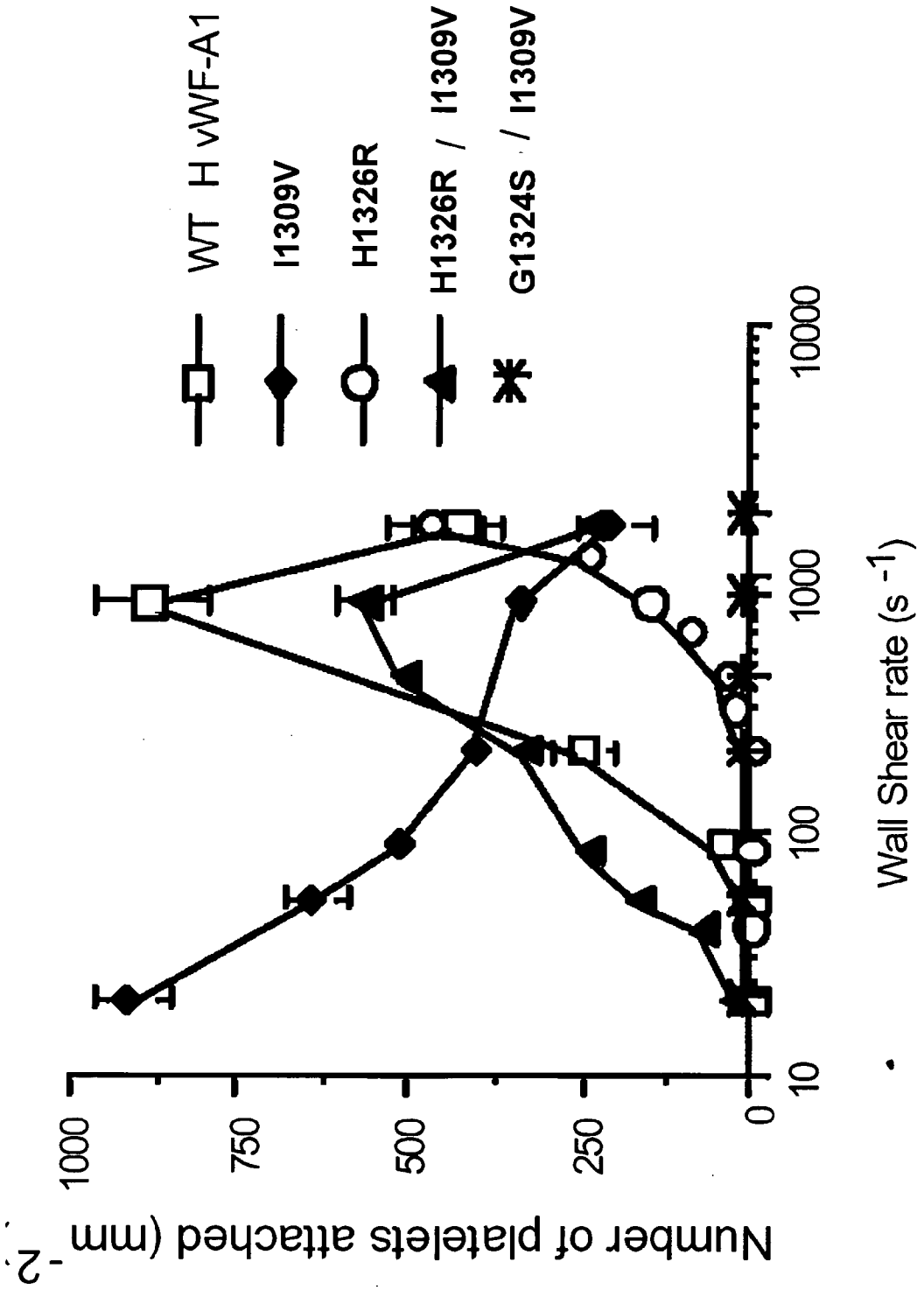

WT H vWF-A1
I1309V
H1326R
H1326R / I1309V
G1324S / I1309V
Number of platelets attached (mm-2)
1000
750
500
250
0
10
100
1000
10000
Wall Shear rate (s-1)

FIG. 19A

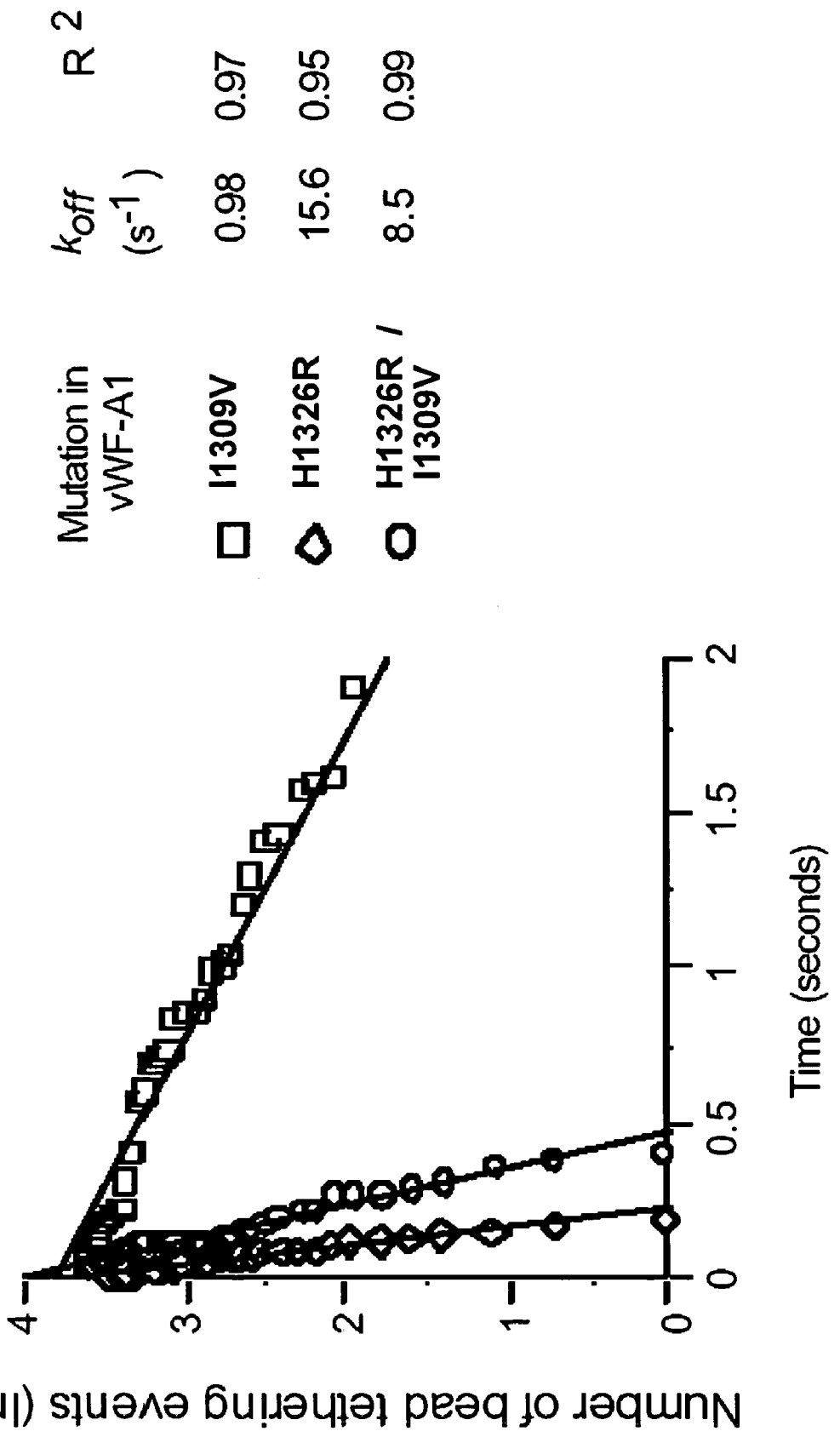
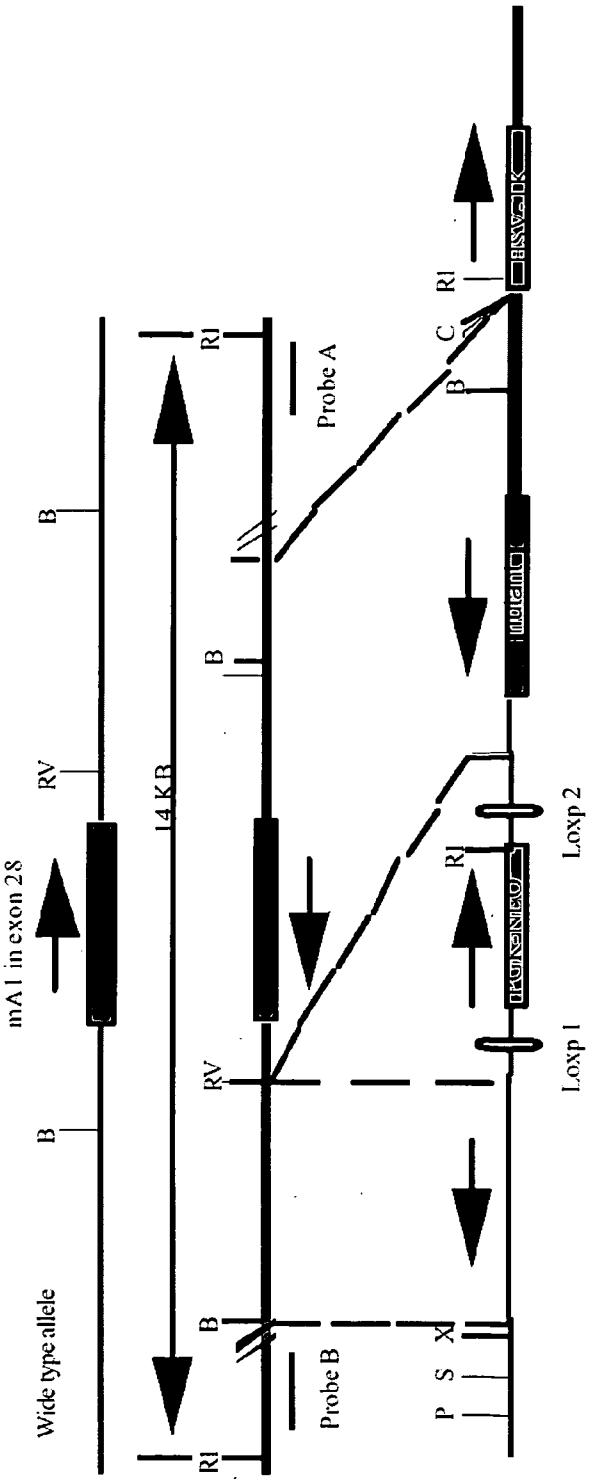
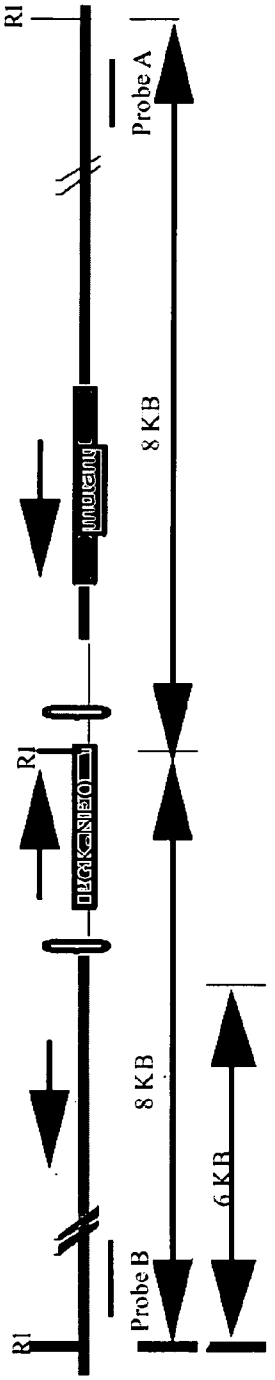

Mutation in vWF-A1
k_{off} (s^{-1})
R^2
I1309V 0.98 0.97
H1326R 15.6 0.95
H1326R / I1309V 8.5 0.99
Number of bead tethering events (ln)
Time (seconds)
0
0.5
1
1.5
2
0
1
2
3
4

FIG. 19B





Knock-in construct for proposed mutations in vWF-A1

FIG. 20A

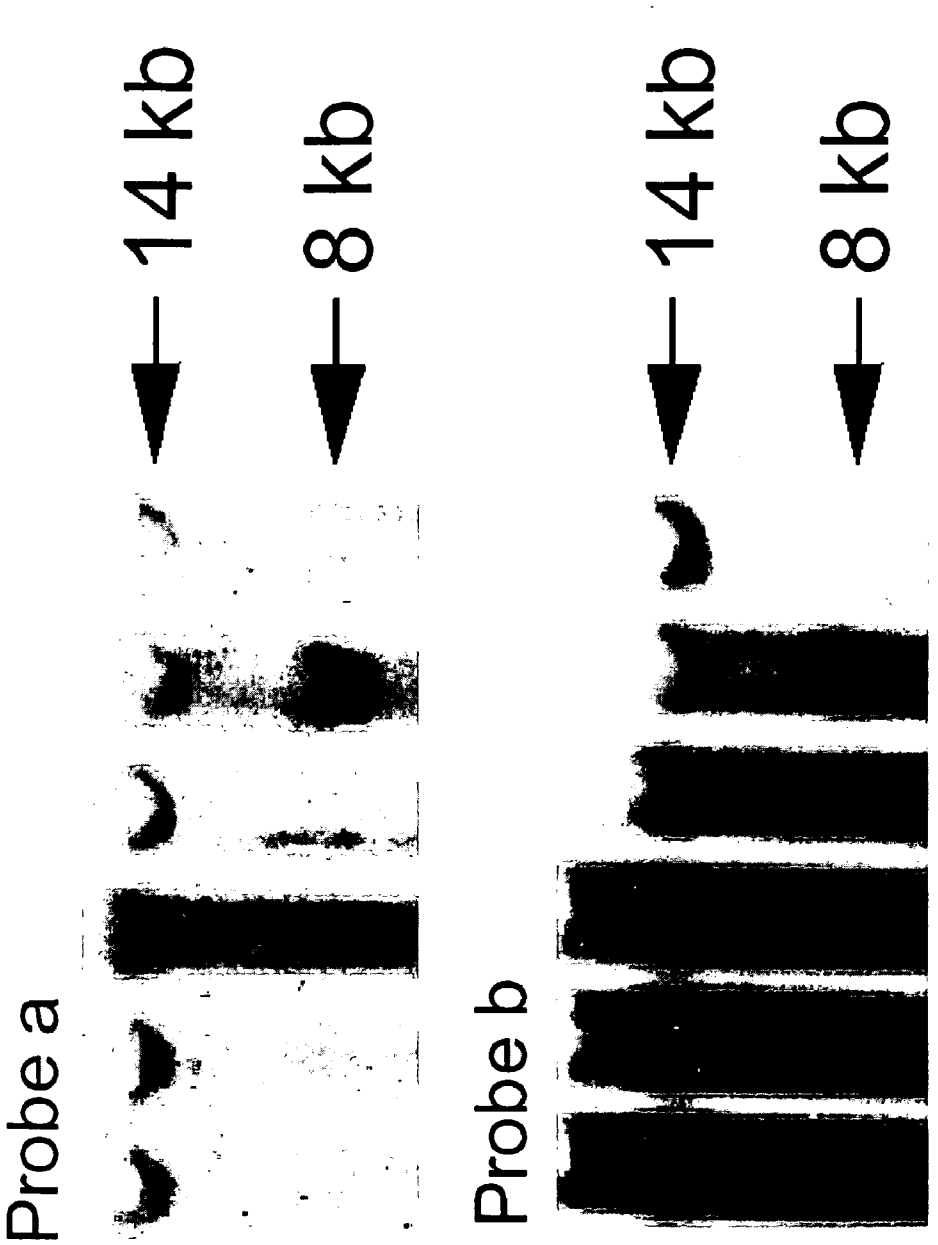


FIG. 20B

FIG. 21

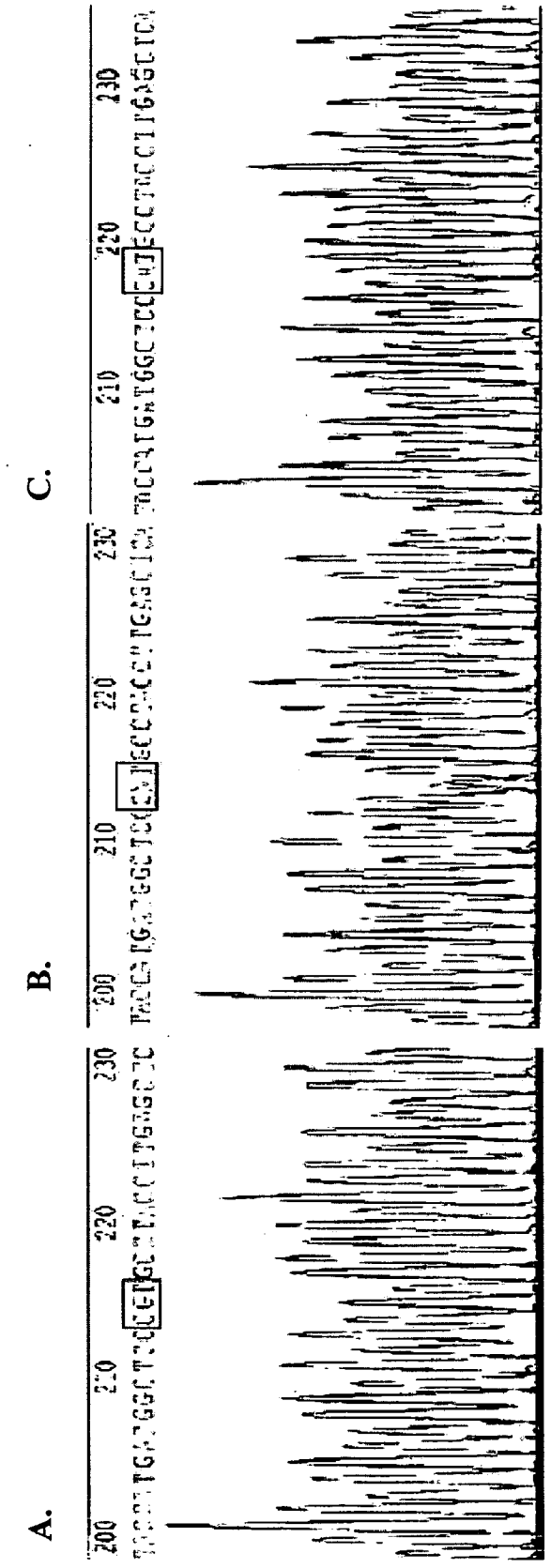


FIG. 22

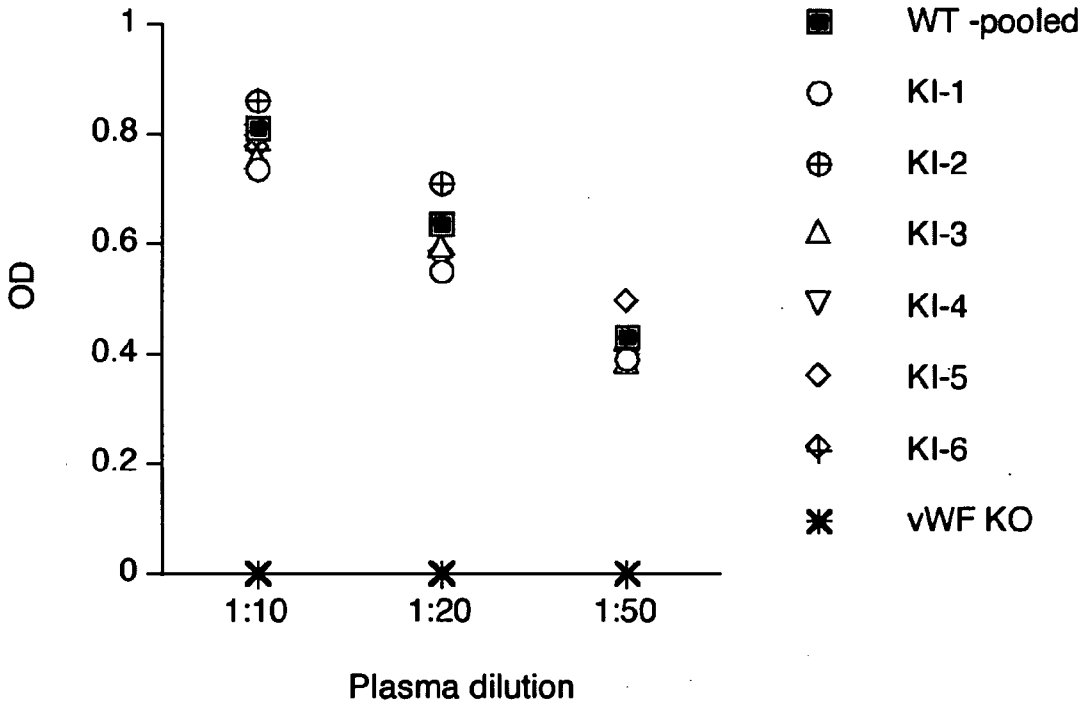

1
0.8
0.6
0.4
0.2
0
OD
1:10
1:20
1:50
Plasma dilution
WT -pooled
KI-1
KI-2
KI-3
KI-4
KI-5
KI-6
vWF KO

FIG. 23

FIG. 24

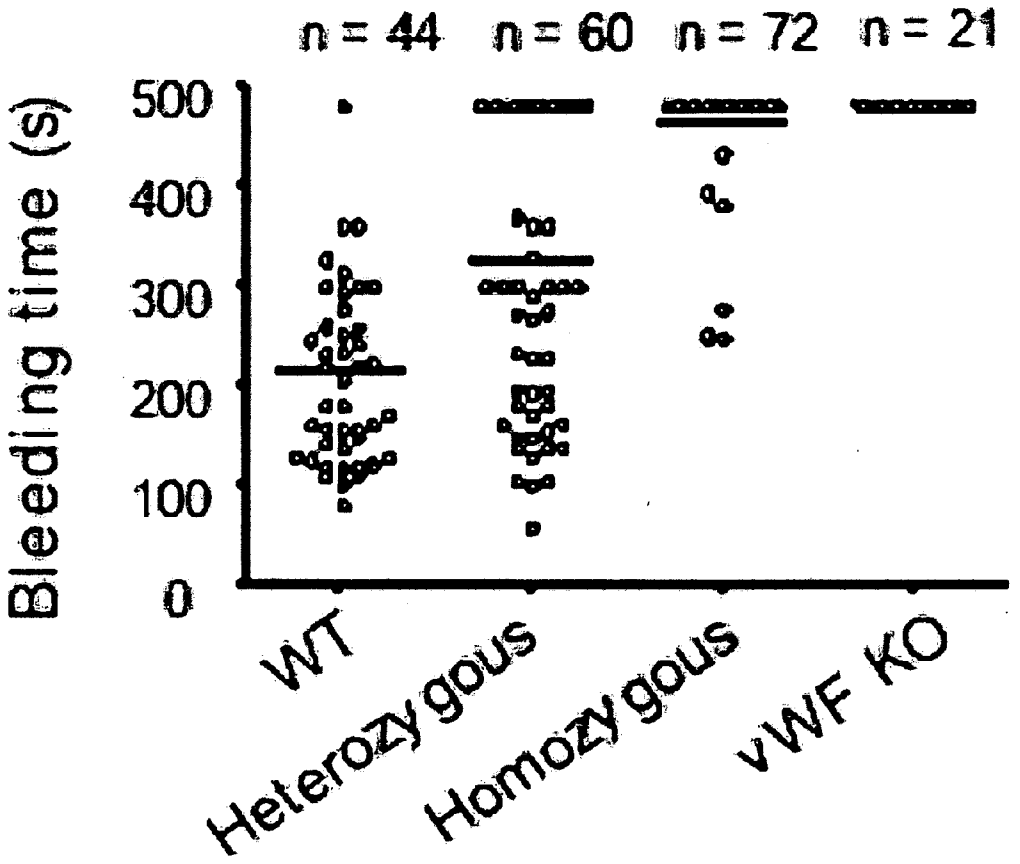

n = 44
n = 60
n = 72
n = 21
Bleeding time (s)
500
400
300
200
100
0
WT
Heterozygous
Homozygous
vWF KO

FIG. 25

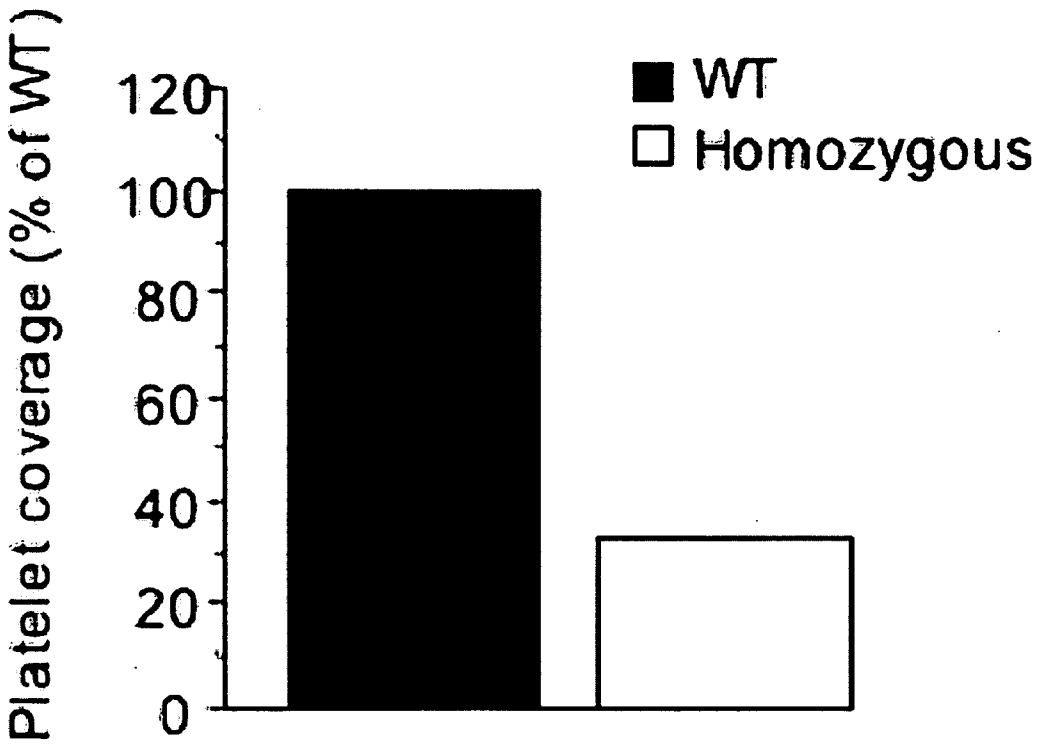


FIG. 26

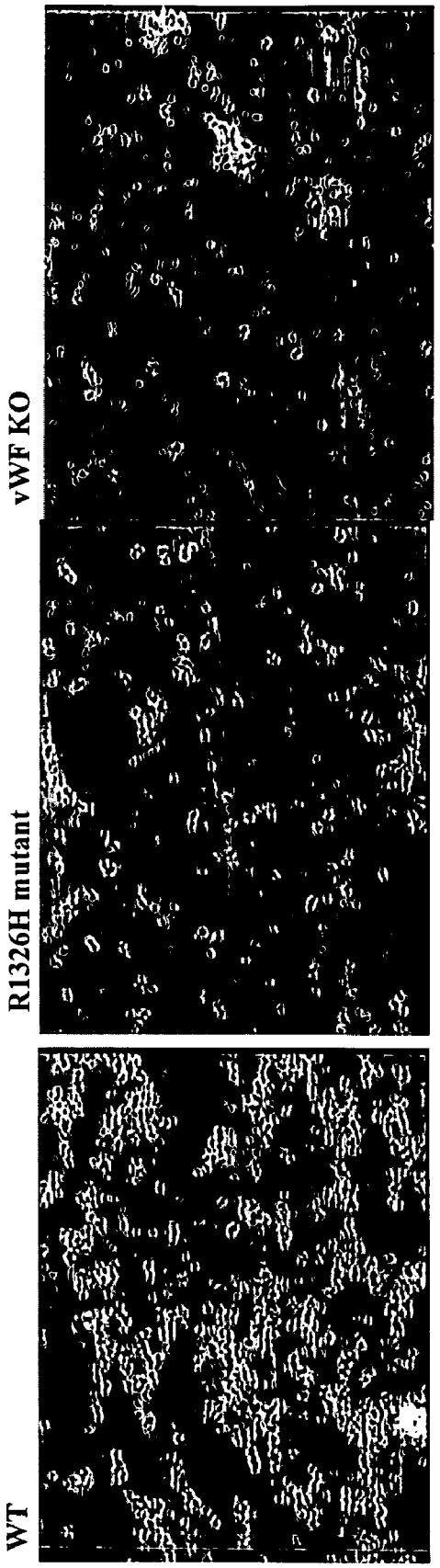


FIG. 27

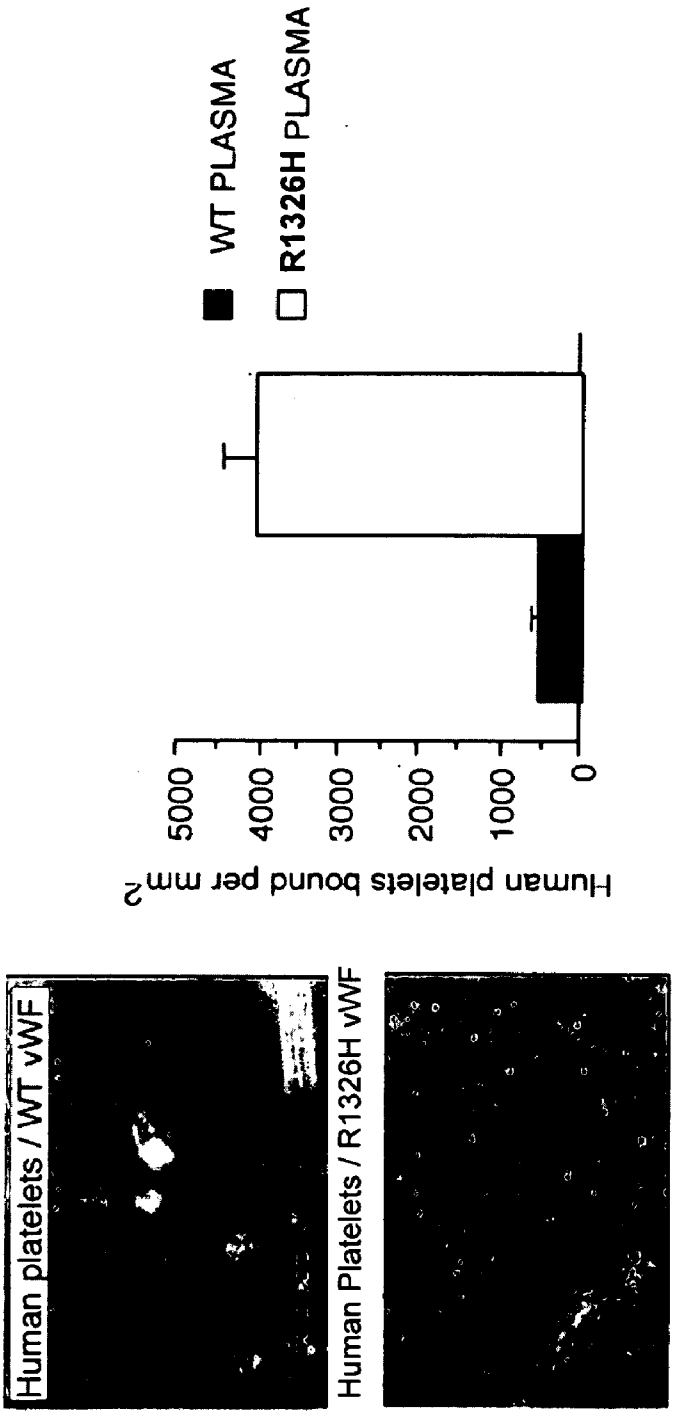
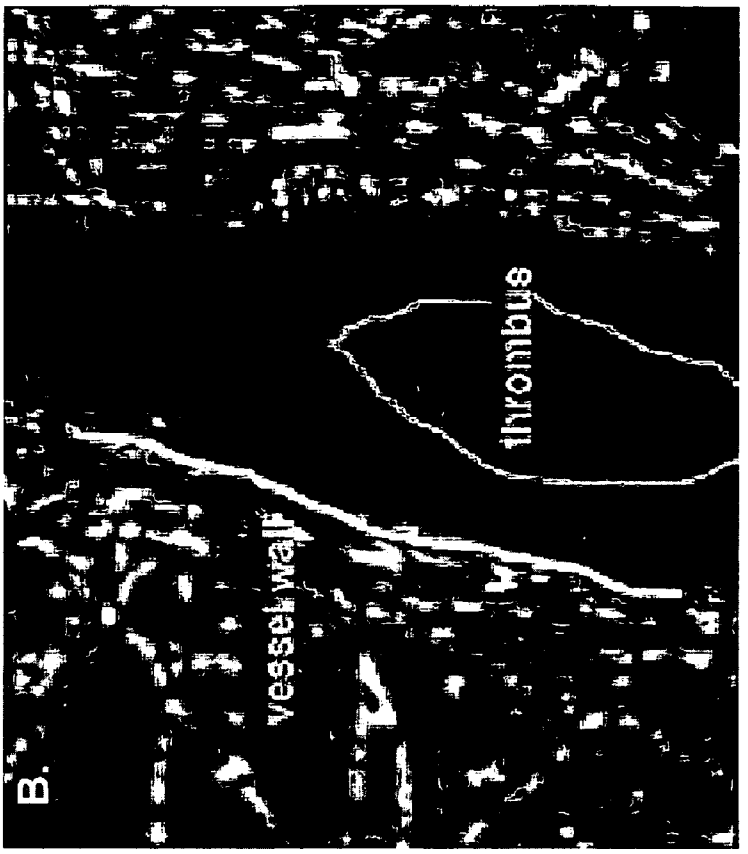

A.
Human platelets / WT vWF
Human Platelets / R1326H vWF
B.
WT PLASMA
R1326H PLASMA
Human platelets bound per mm2
5000
4000
3000
2000
1000
0

FIG. 28



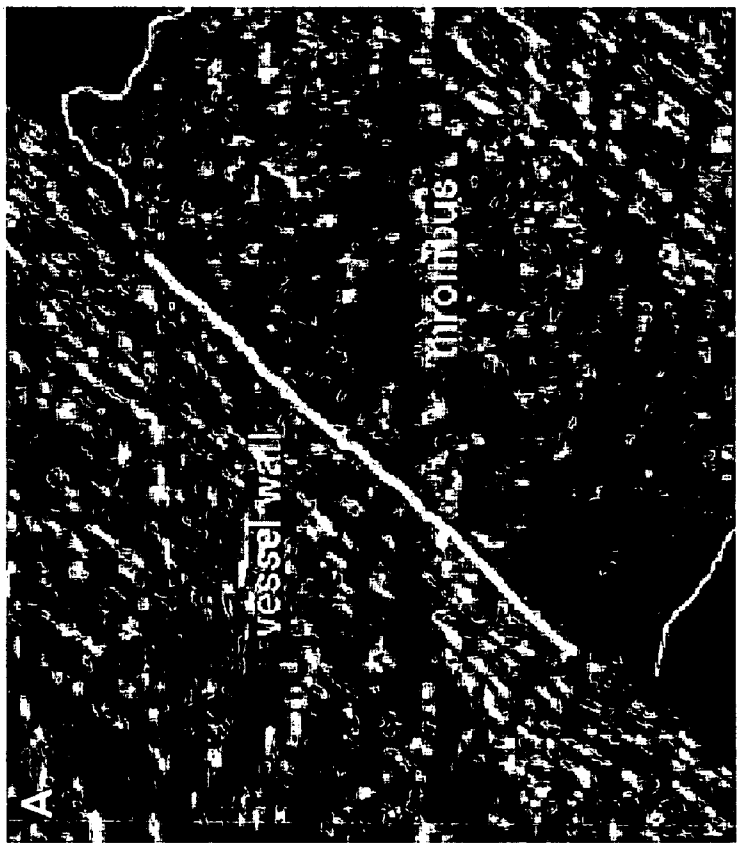


FIG. 29

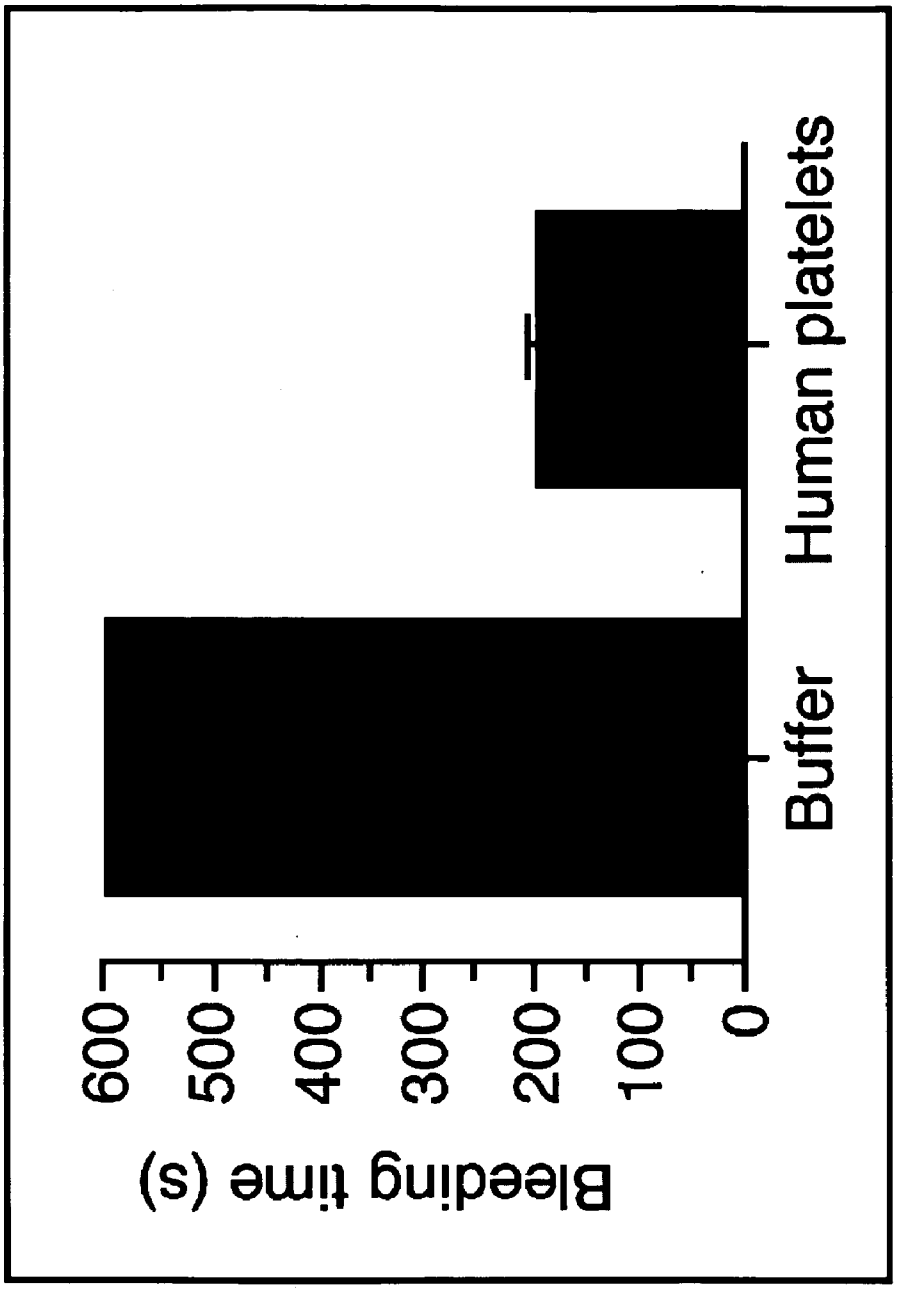

Bleeding time (s)
600
500
400
300
200
100
0
Buffer
Human platelets

FIG. 30

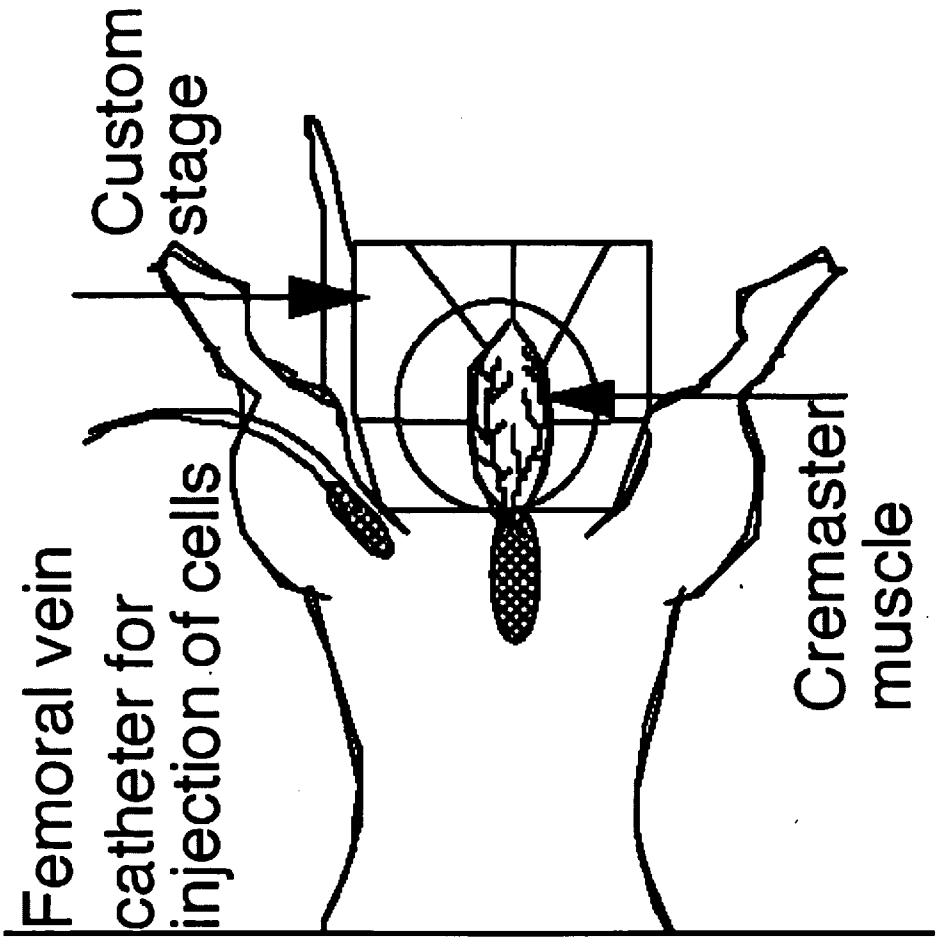

Custom stage
Femoral vein catheter for injection of cells
Cremaster muscle

FIG. 31

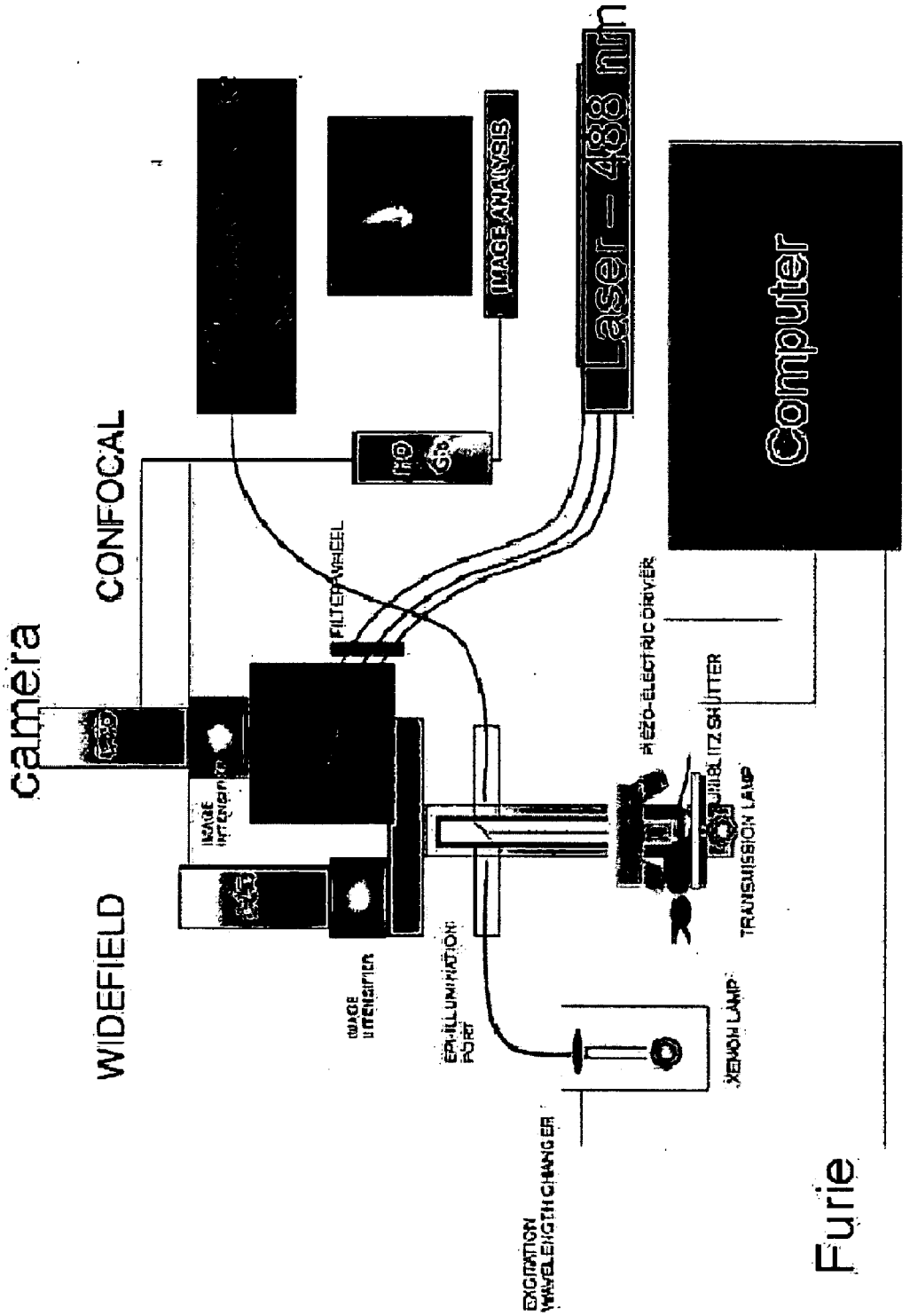

camera
CONFOCAL
WIDEFIELD
IMAGE ANALYSIS
Laser – 488 nm
Computer
Furie
FILTER WHEEL
PIEZO-ELECTRIC DRIVER
TRANSMISSION LAMP
XENON LAMP
EXCITATION WAVELENGTH CHANGER

FIG. 32

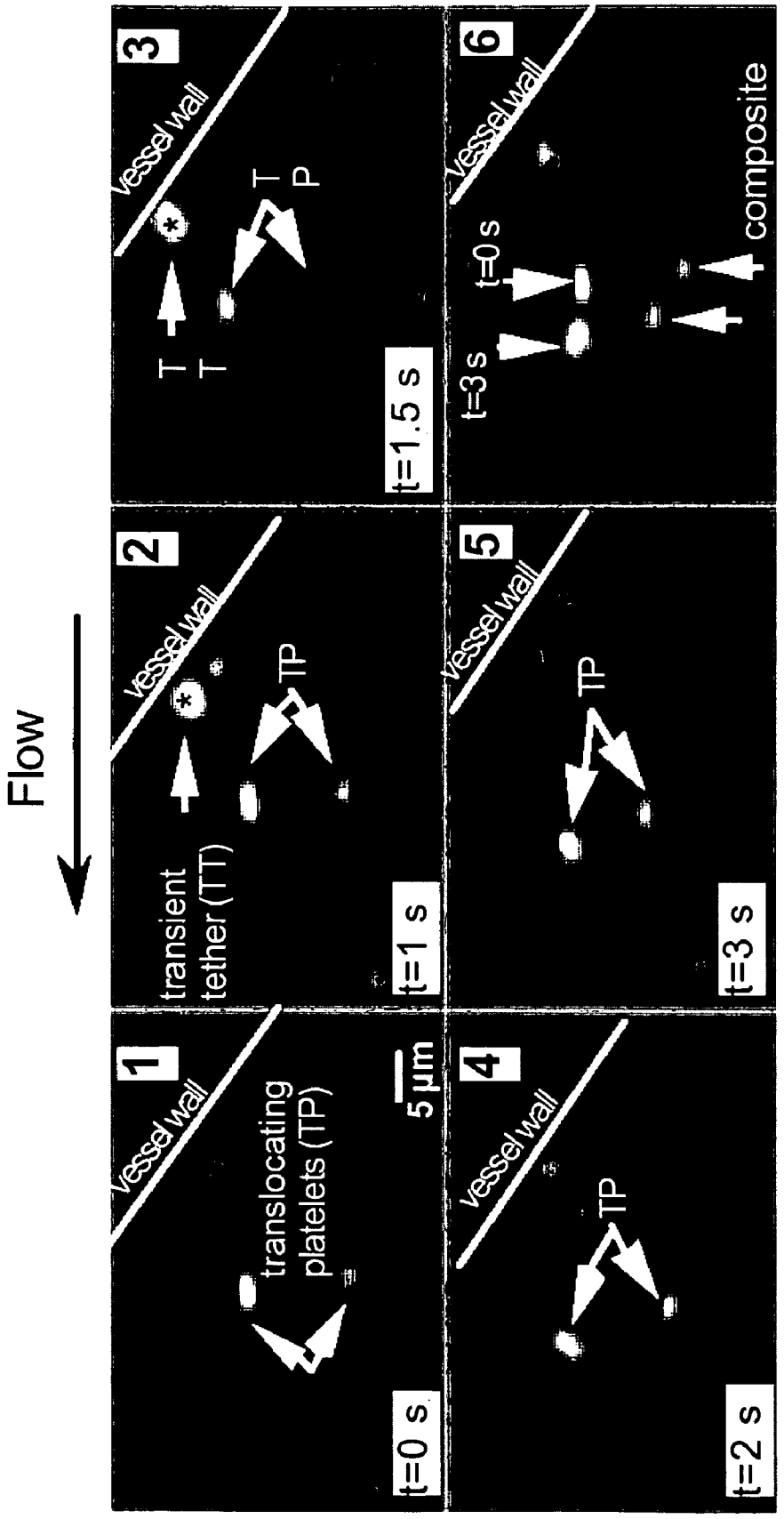

Flow
1
vessel wall
translocating platelets (TP)
5 µm
t=0 s
2
vessel wall
transient tether (TT)
TP
t=1 s
3
vessel wall
T
T
T
P
t=1.5 s
4
vessel wall
TP
t=2 s
5
vessel wall
TP
t=3 s
6
vessel wall
t=0 s
t=3 s
composite

FIG. 33A

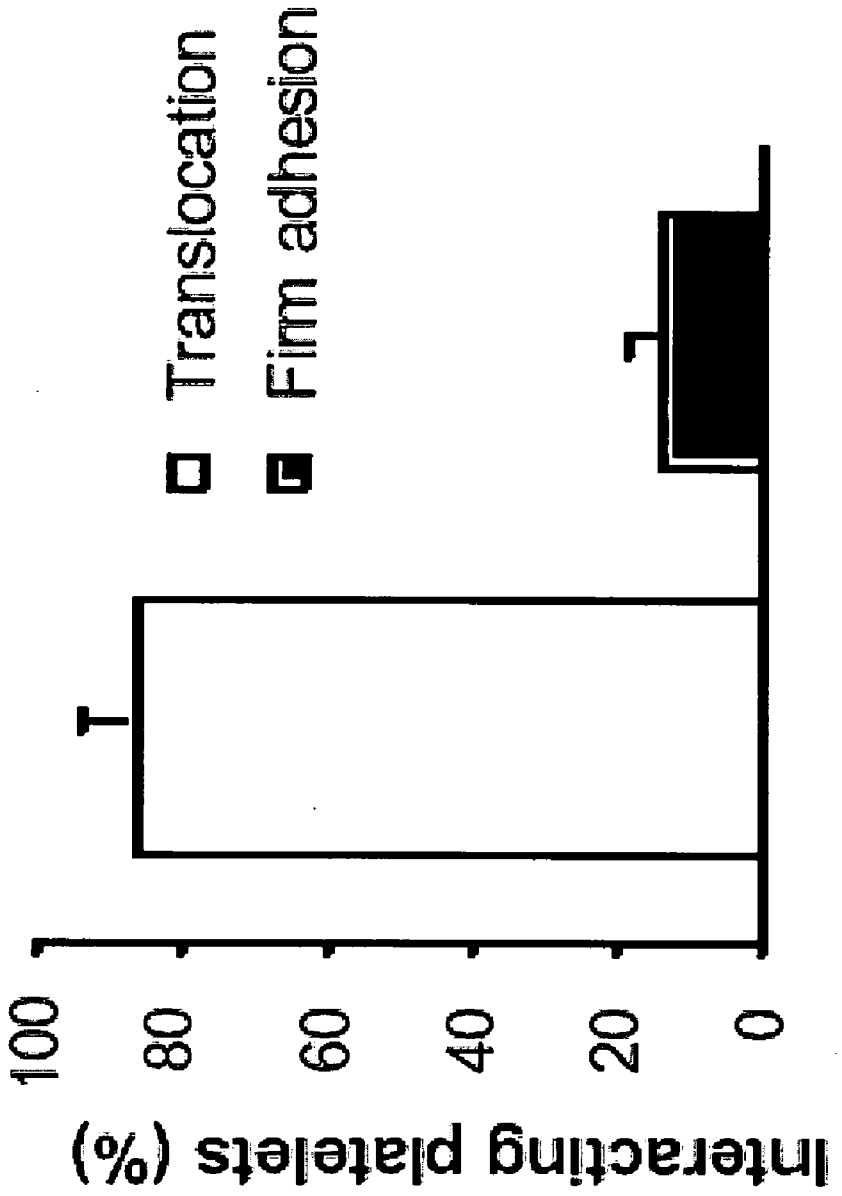

Translocation
Firm adhesion
Interacting platelets (%)
100
80
60
40
20
0

FIG. 33B

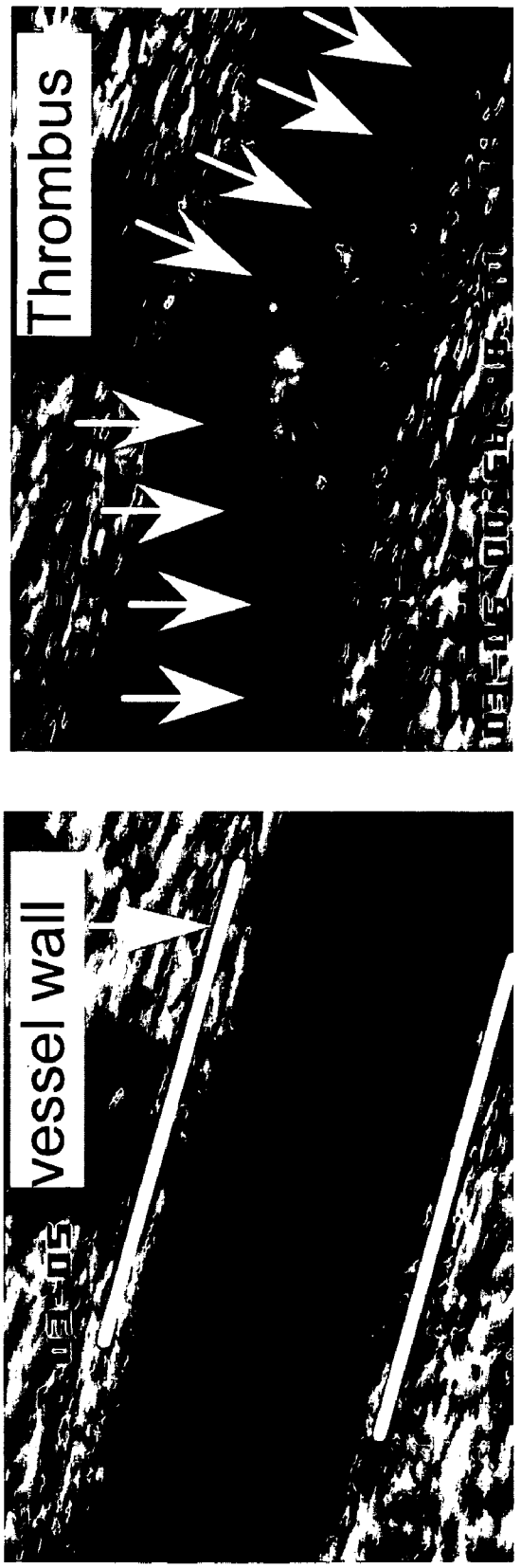


FIG. 34

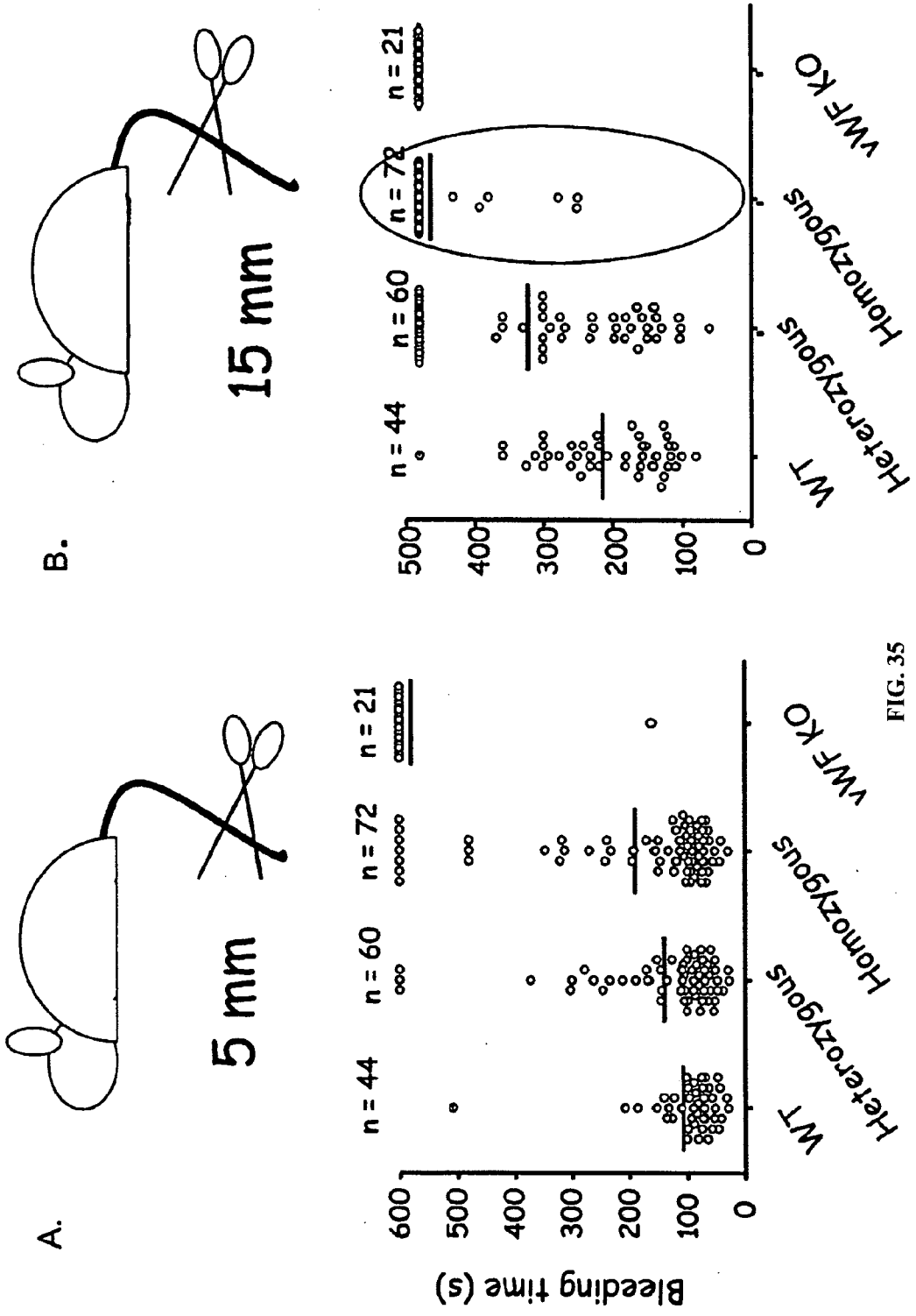

A.
5 mm
B.
15 mm
Bleeding time (s)
0
100
200
300
400
500
600
n = 44
n = 60
n = 72
n = 21
WT
Heterozygous
Homozygous
vWF KO

FIG. 35

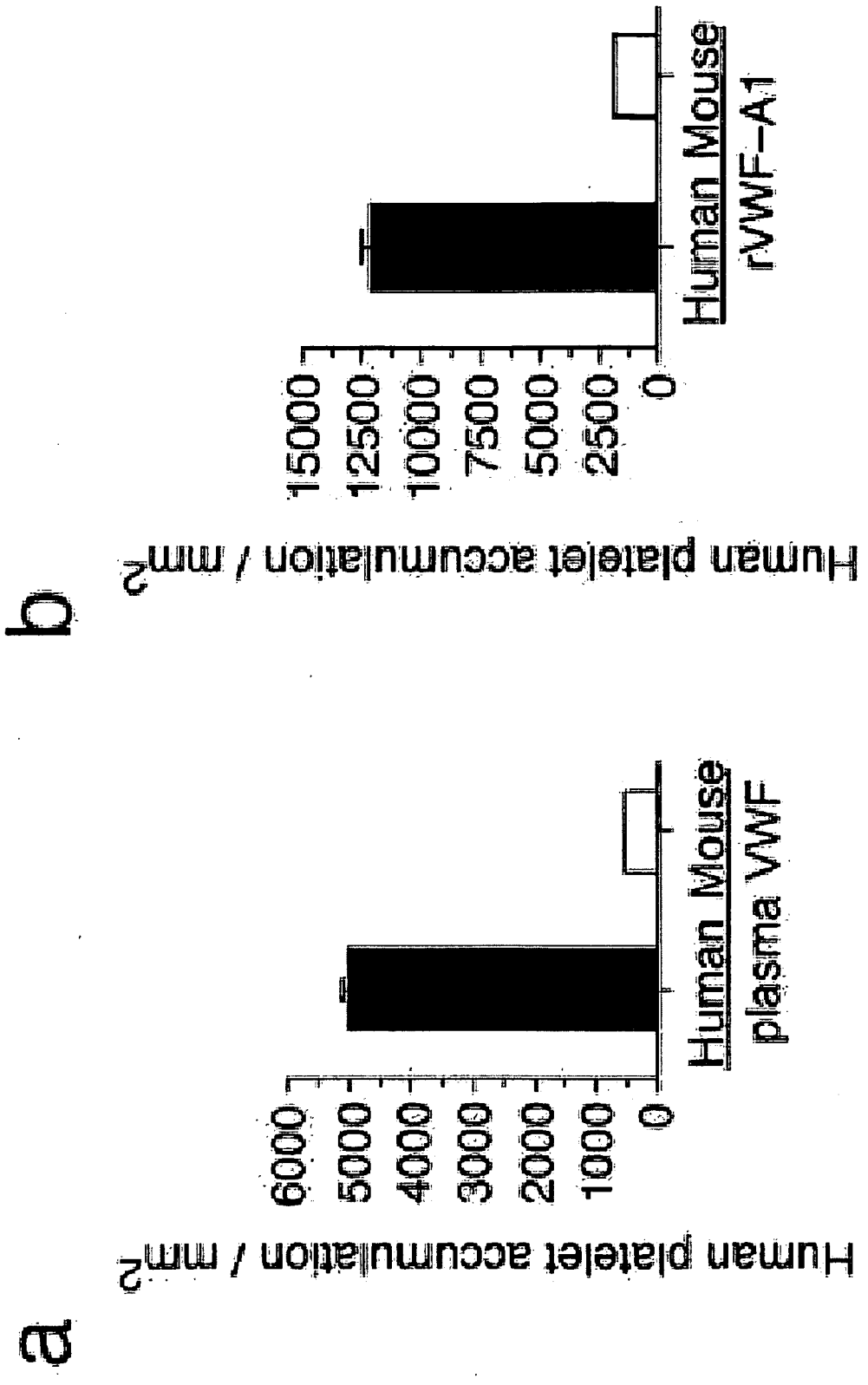

a
Human platelet accumulation / mm²
6000
5000
4000
3000
2000
1000
0
Human Mouse
plasma VWF
b
Human platelet accumulation / mm²
15000
12500
10000
7500
5000
2500
0
Human Mouse
rVWF-A1

FIG. 36A-B

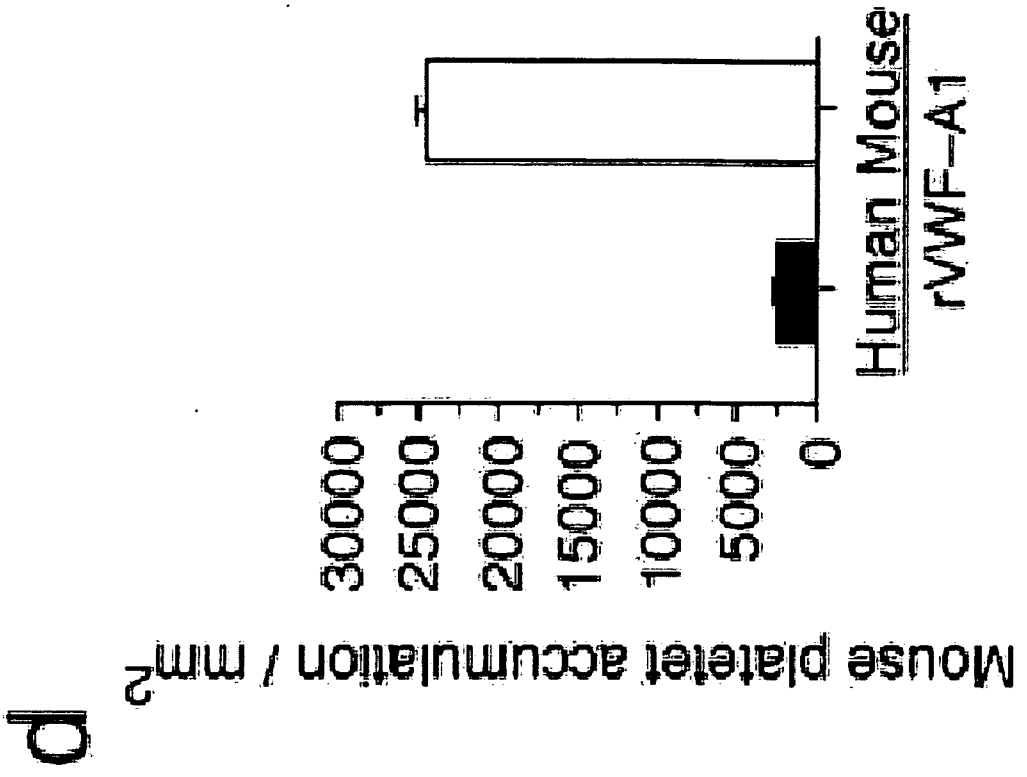

C
Mouse platelet accumulation / mm2
25000
20000
15000
10000
5000
0
Human Mouse
plasma VWF
d
Mouse platelet accumulation / mm2
30000
25000
20000
15000
10000
5000
0
Human Mouse
rVWF–A1

FIG. 36C-D

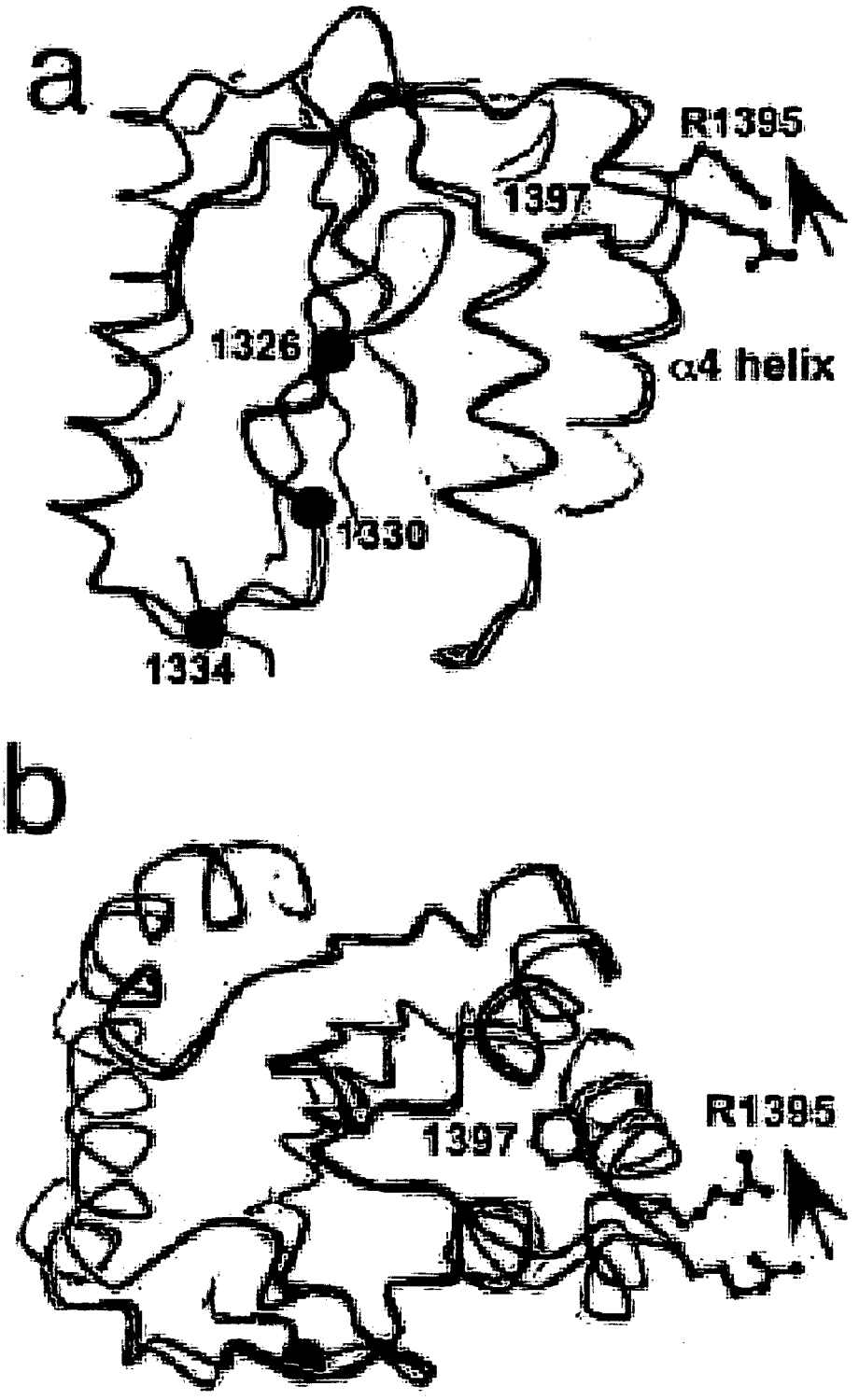


FIG. 37A-B

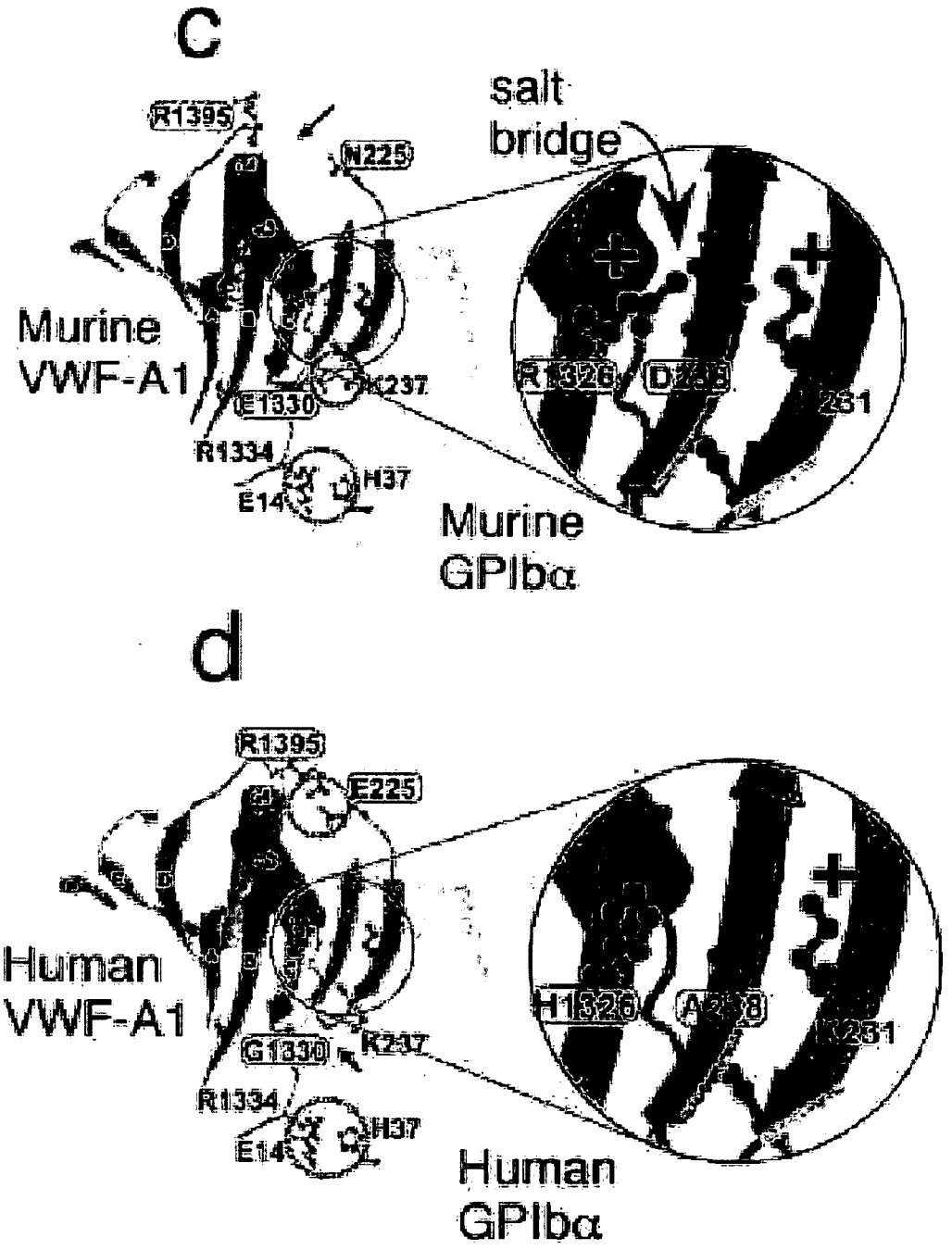

c
salt
bridge
R1395
N225
Murine
VWF-A1
E1330
K237
R1334
H37
E14
R1326
D239
K231
Murine
GPIbα
d
R1395
E225
Human
VWF-A1
G1330
K237
R1334
H37
E14
H1326
K231
Human
GPIbα

FIG. 37C-D

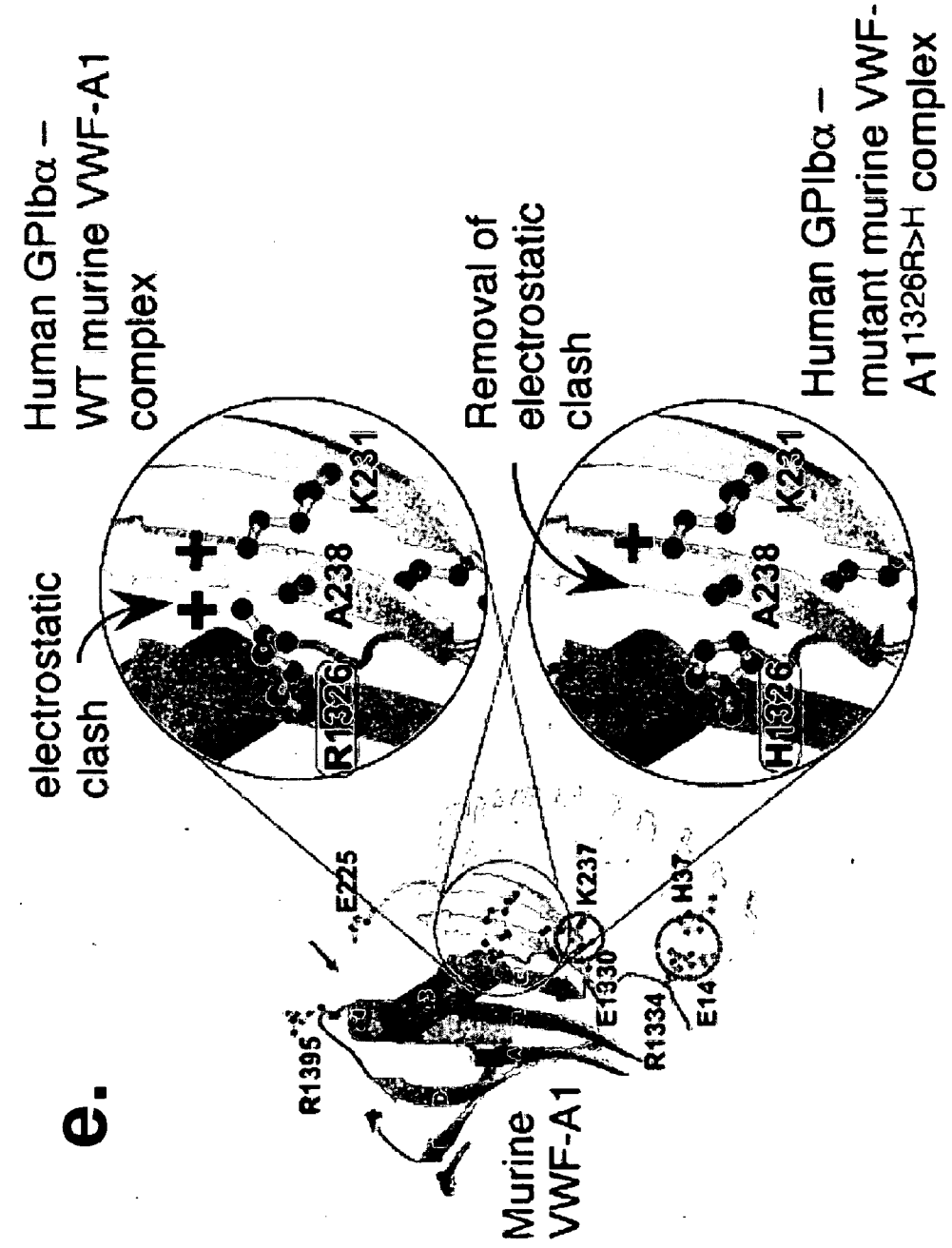

e.
Murine VWF-A1
R1395
E225
K237
E1330
R1334
H37
E14
electrostatic clash
Human GPIbα – WT murine VWF-A1 complex
R1326
A238
K231
Removal of electrostatic clash
Human GPIbα – mutant murine VWF-A1$^{1326R>H}$ complex
H1326
A238
K231

FIG. 37E

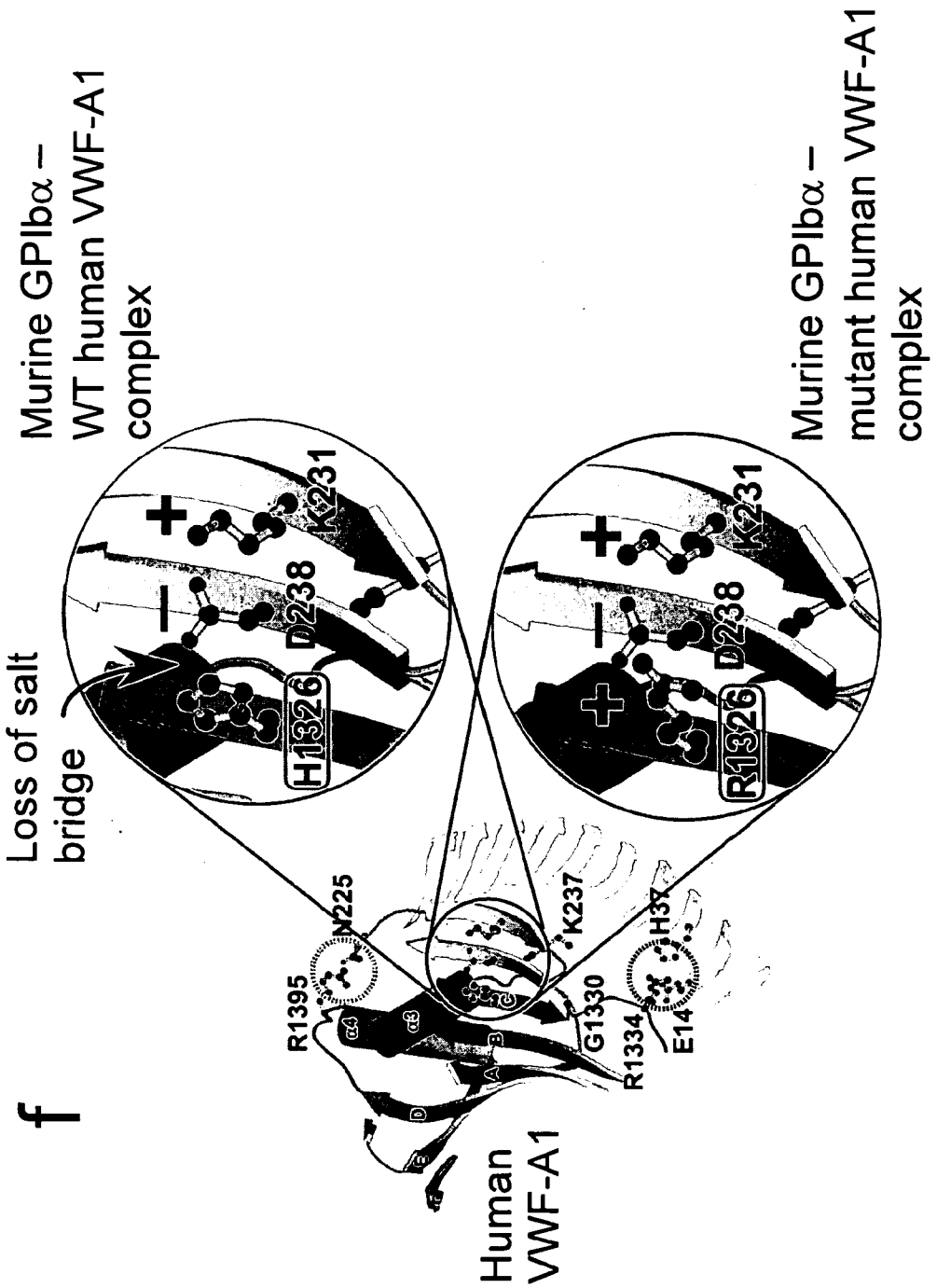

f
Loss of salt bridge
Murine GPIbα – WT human VWF-A1 complex
Murine GPIbα – mutant human VWF-A1 complex
Human VWF-A1
H1326
D238
K231
R1326
R1395
K225
K237
G1330
R1334
E14
H37

FIG. 37F

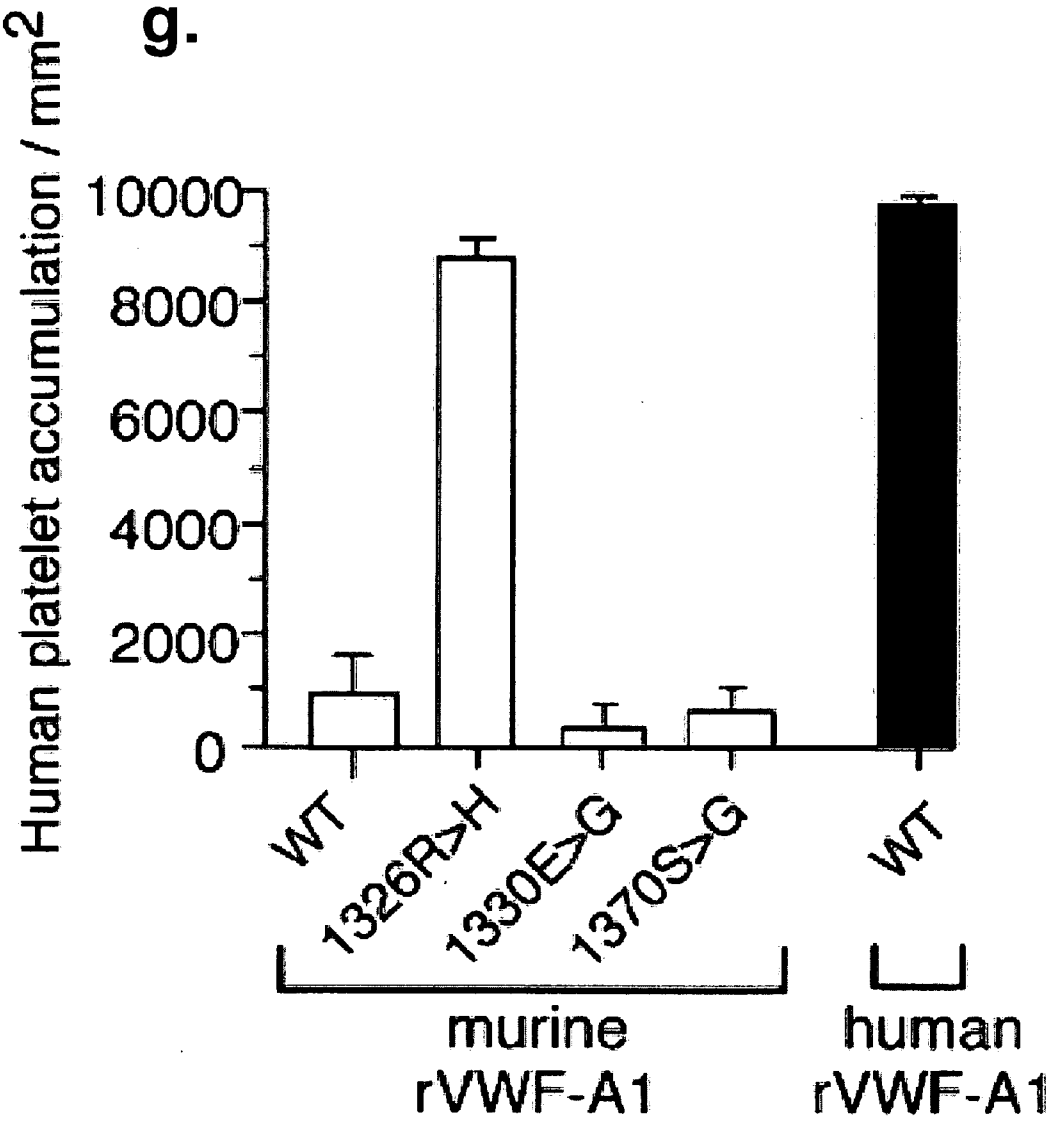

g.
Human platelet accumulation / mm2
10000
8000
6000
4000
2000
0
WT
1326R>H
1330E>G
1370S>G
WT
murine rVWF-A1
human rVWF-A1

FIG. 37G

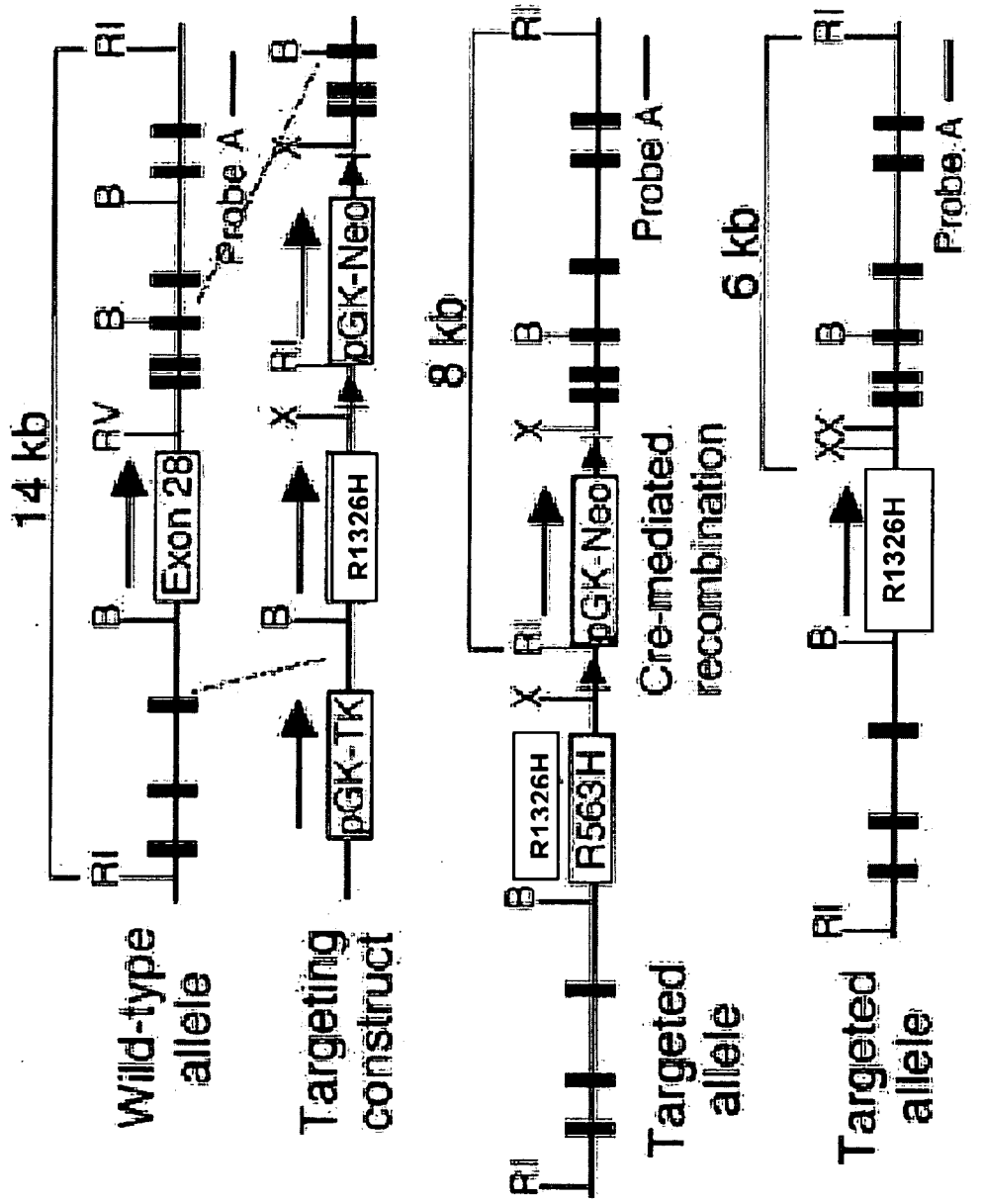

14 kb
RI
RV
B
B
B
Exon 28
Probe A
Wild-type allele
Targeting construct
pGK-TK
R1326H
X
RI
pGK-Neo
8 kb
R1326H
R563H
Cre-mediated recombination
Targeted allele
6 kb
XX
Targeted allele

FIG. 38A

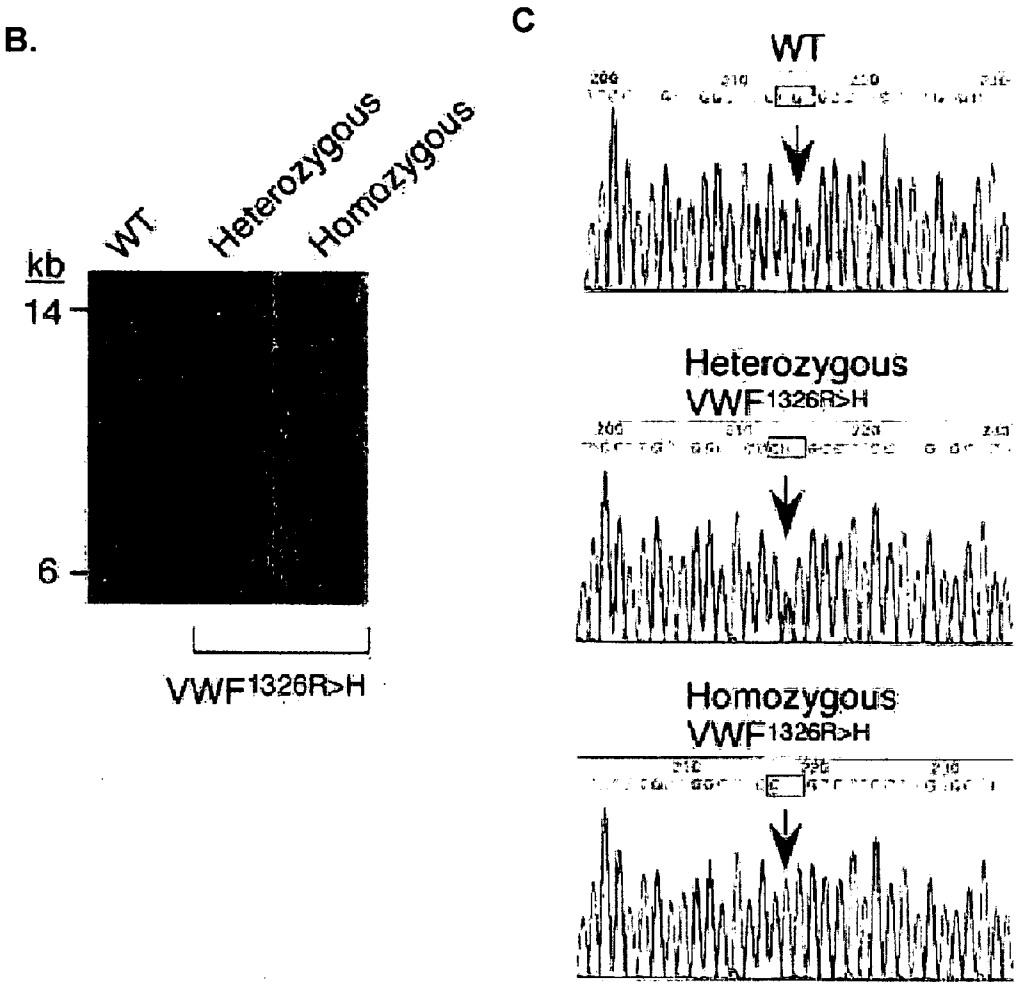
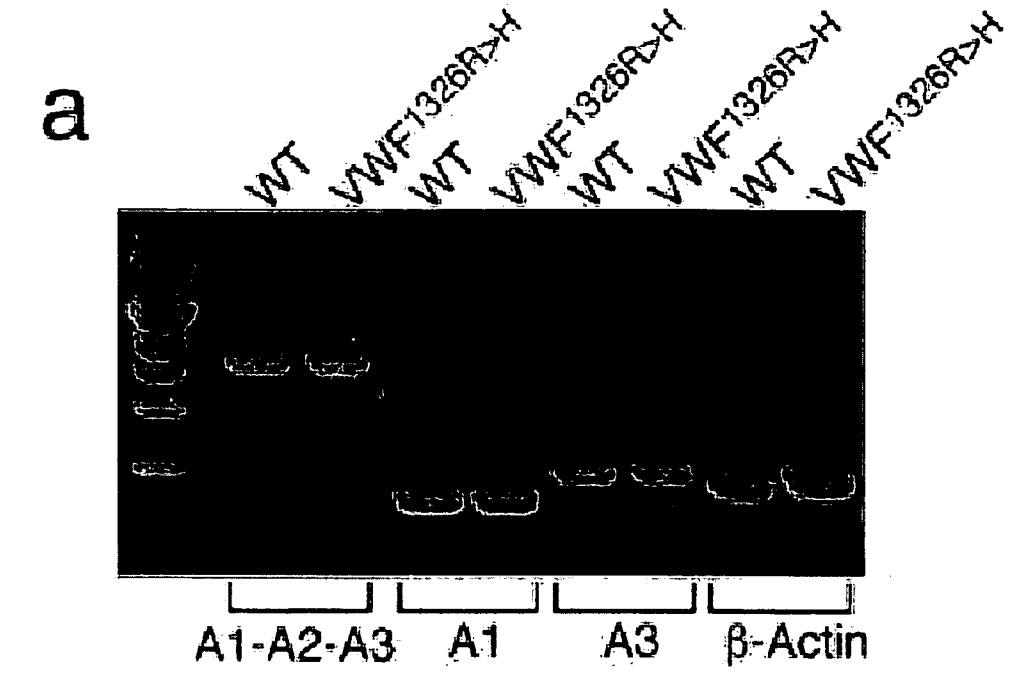

B.
WT
Heterozygous
Homozygous
kb
14
6
VWF1326R>H
C
WT
Heterozygous
VWF1326R>H
Homozygous
VWF1326R>H

FIG. 38B-C


a
WT
VWF1326R>H
WT
VWF1326R>H
WT
VWF1326R>H
WT
VWF1326R>H
A1-A2-A3
A1
A3
β-Actin

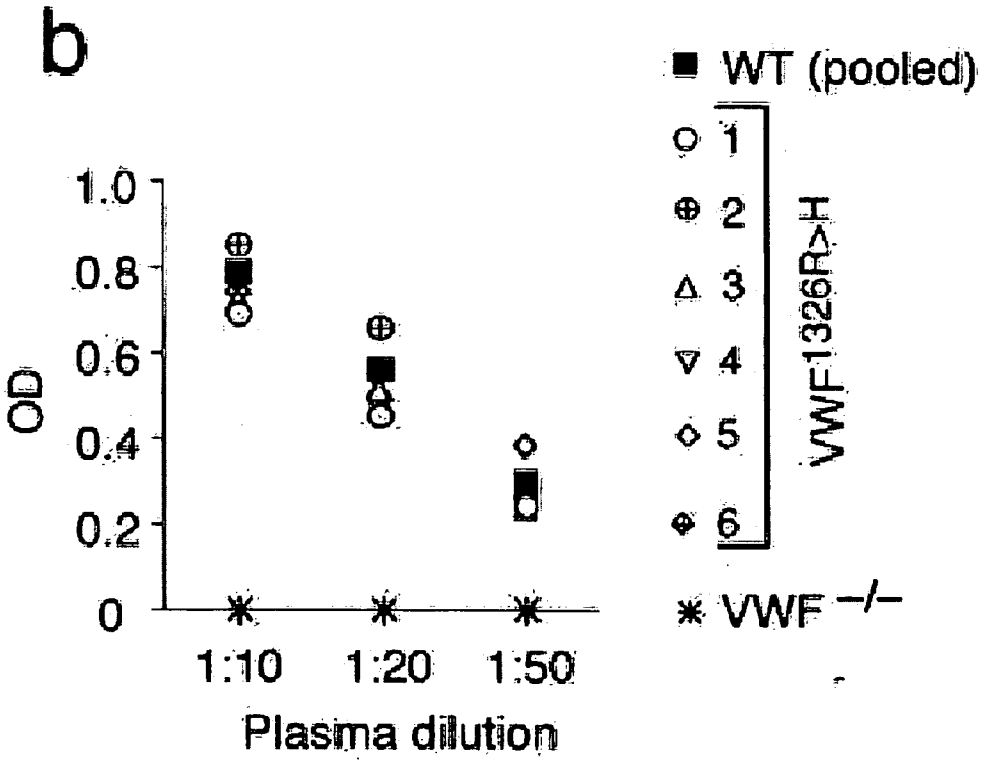

b
WT (pooled)
1
2
3
4
5
6
VWF1326R>H
VWF −/−
OD
1.0
0.8
0.6
0.4
0.2
0
1:10
1:20
1:50
Plasma dilution

FIG. 39A-B

FIG. 39C

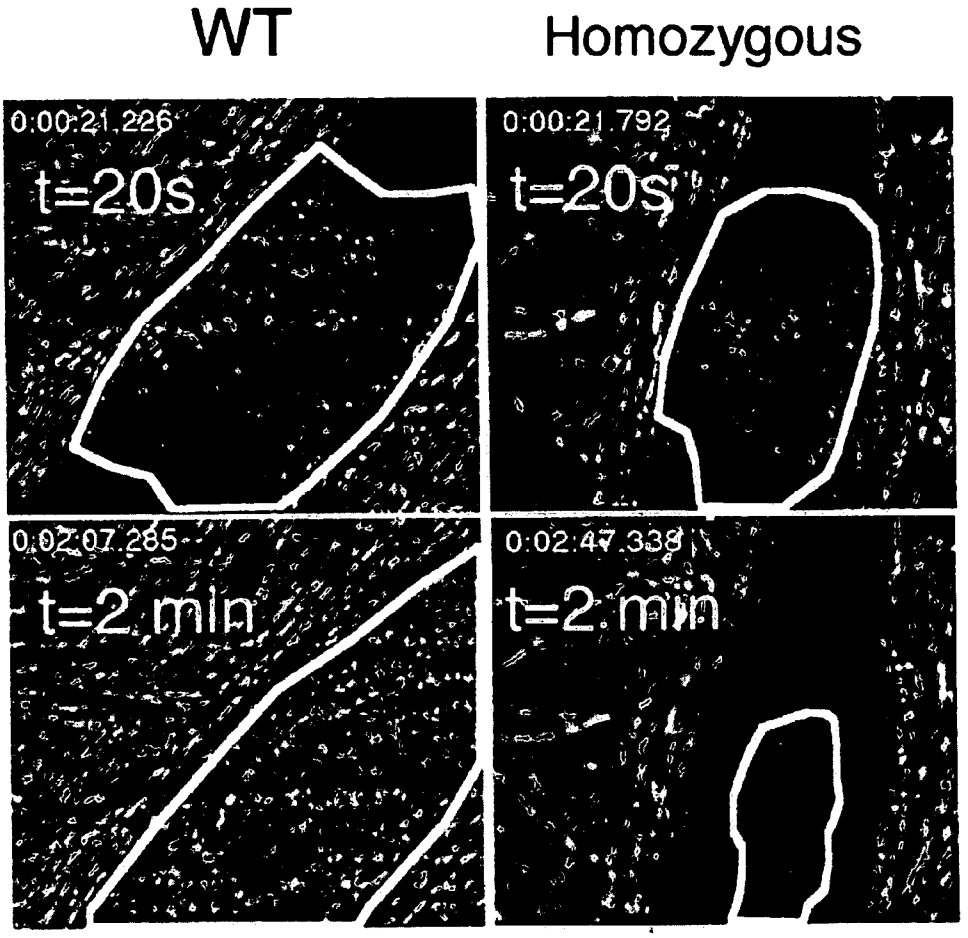

WT
Homozygous
0:00:21.226
t=20s
0:00:21.792
t=20s
0:02:07.285
t=2 min
0:02:47.338
t=2 min

FIG. 40

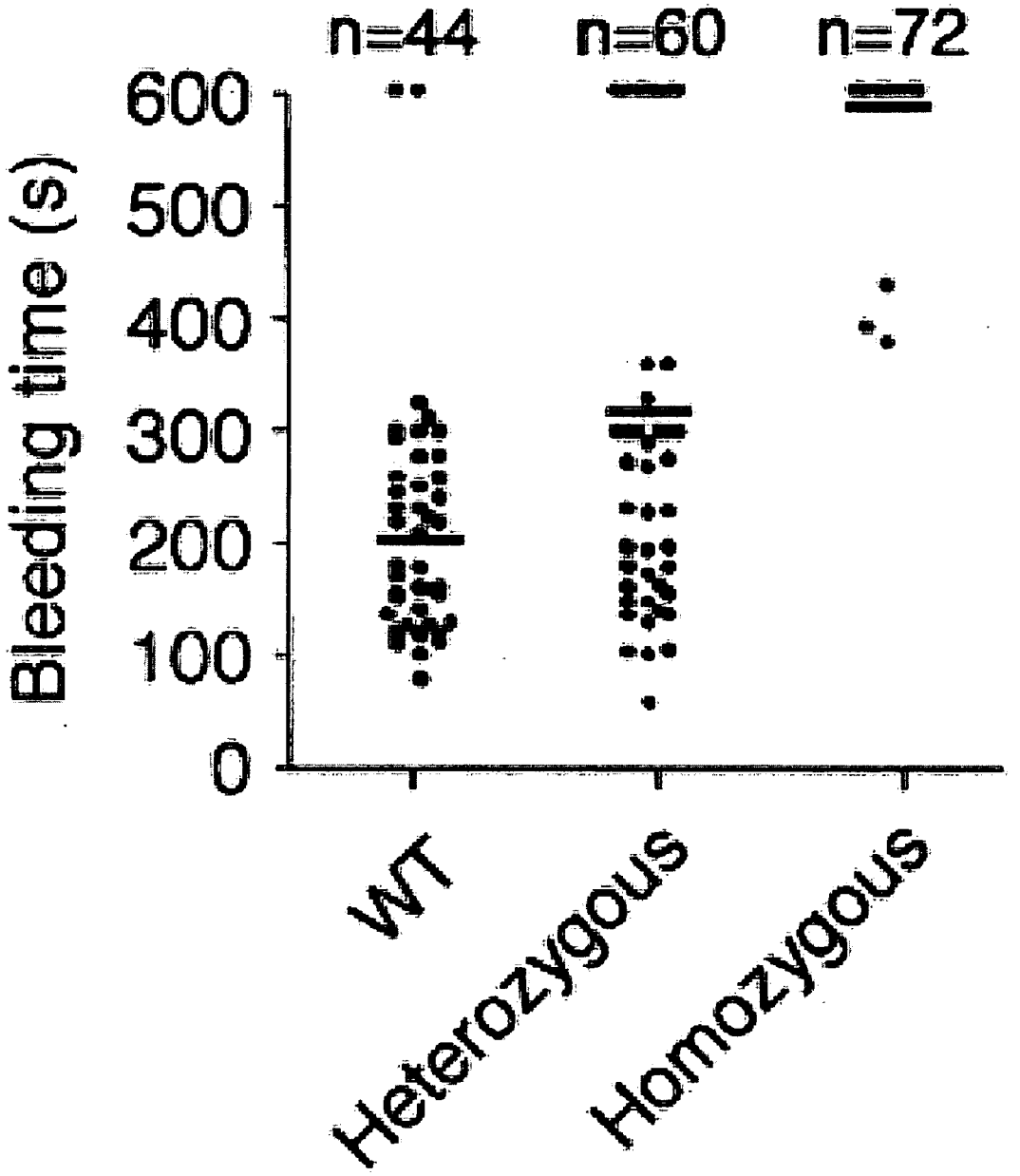

n=44
n=60
n=72
Bleeding time (s)
600
500
400
300
200
100
0
WT
Heterozygous
Homozygous

FIG. 41A

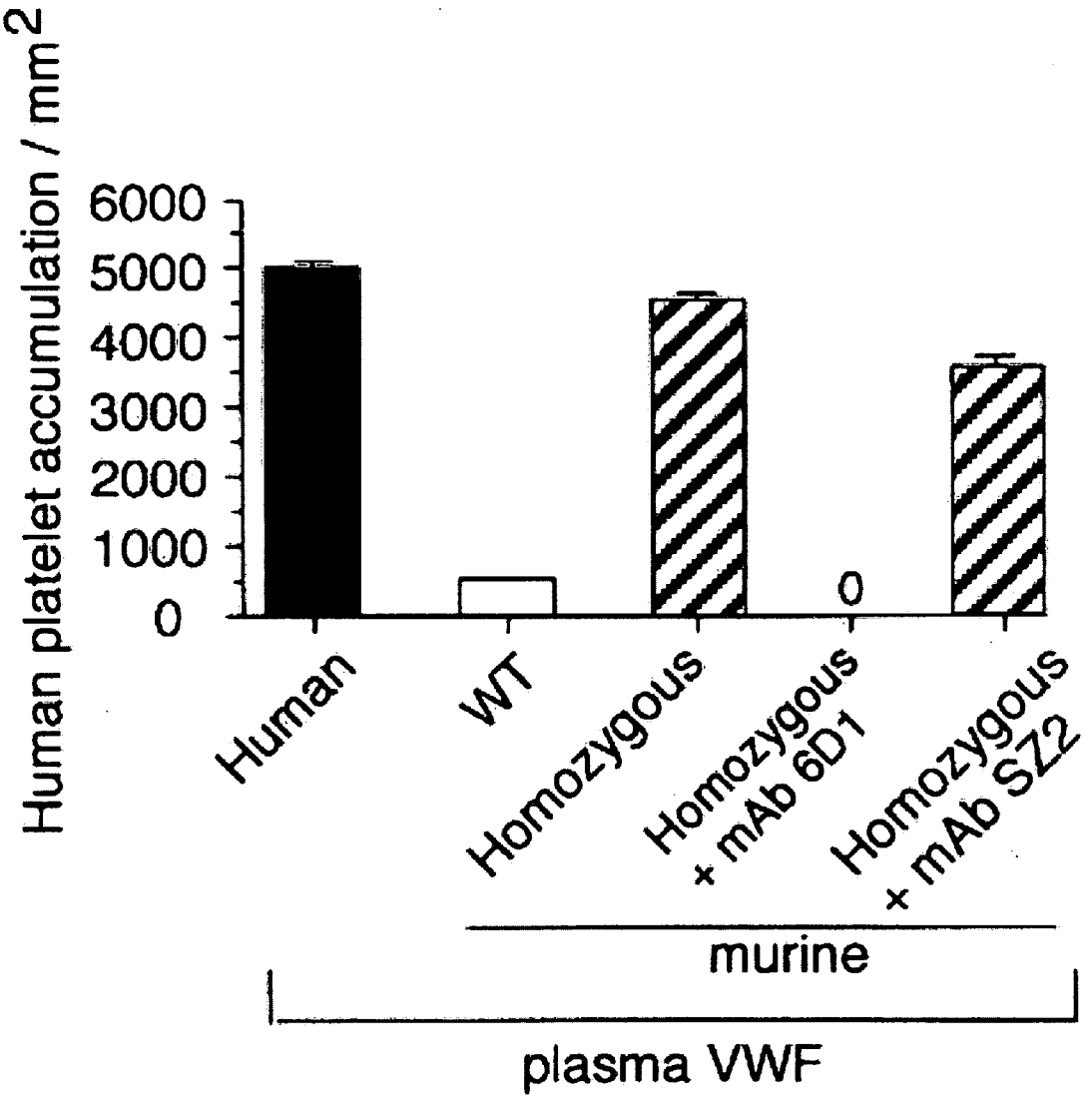

Human platelet accumulation / mm2
6000
5000
4000
3000
2000
1000
0
0
Human
WT
Homozygous
Homozygous + mAb 6D1
Homozygous + mAb SZ2
murine
plasma VWF

FIG. 41B

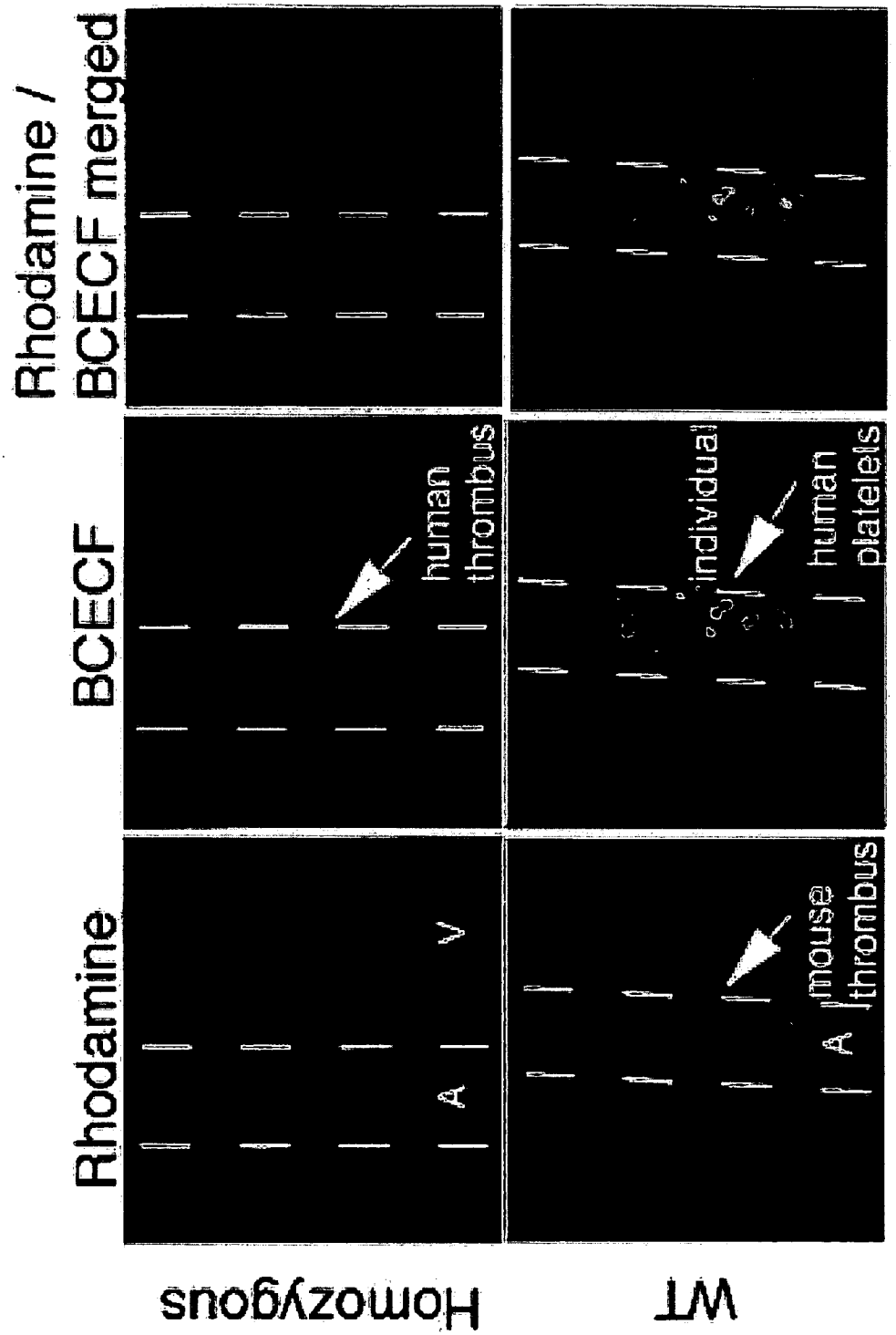

Rhodamine
BCECF
Rhodamine / BCECF merged
Homozygous
WT
A
V
human thrombus
mouse thrombus
individual
human platelets

FIG. 41C

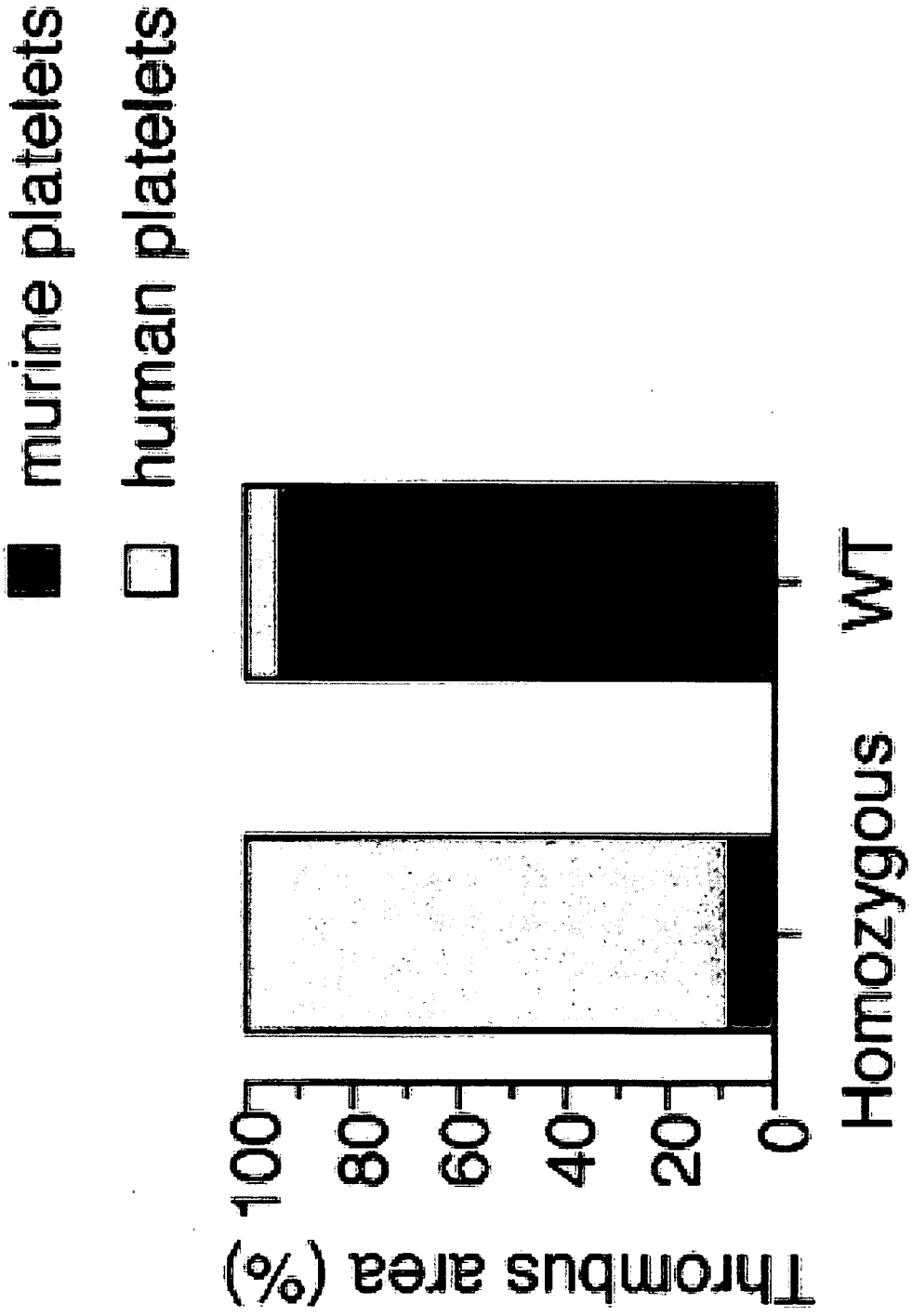

murine platelets
human platelets
Thrombus area (%)
100
80
60
40
20
0
Homozygous
WT

FIG. 41D

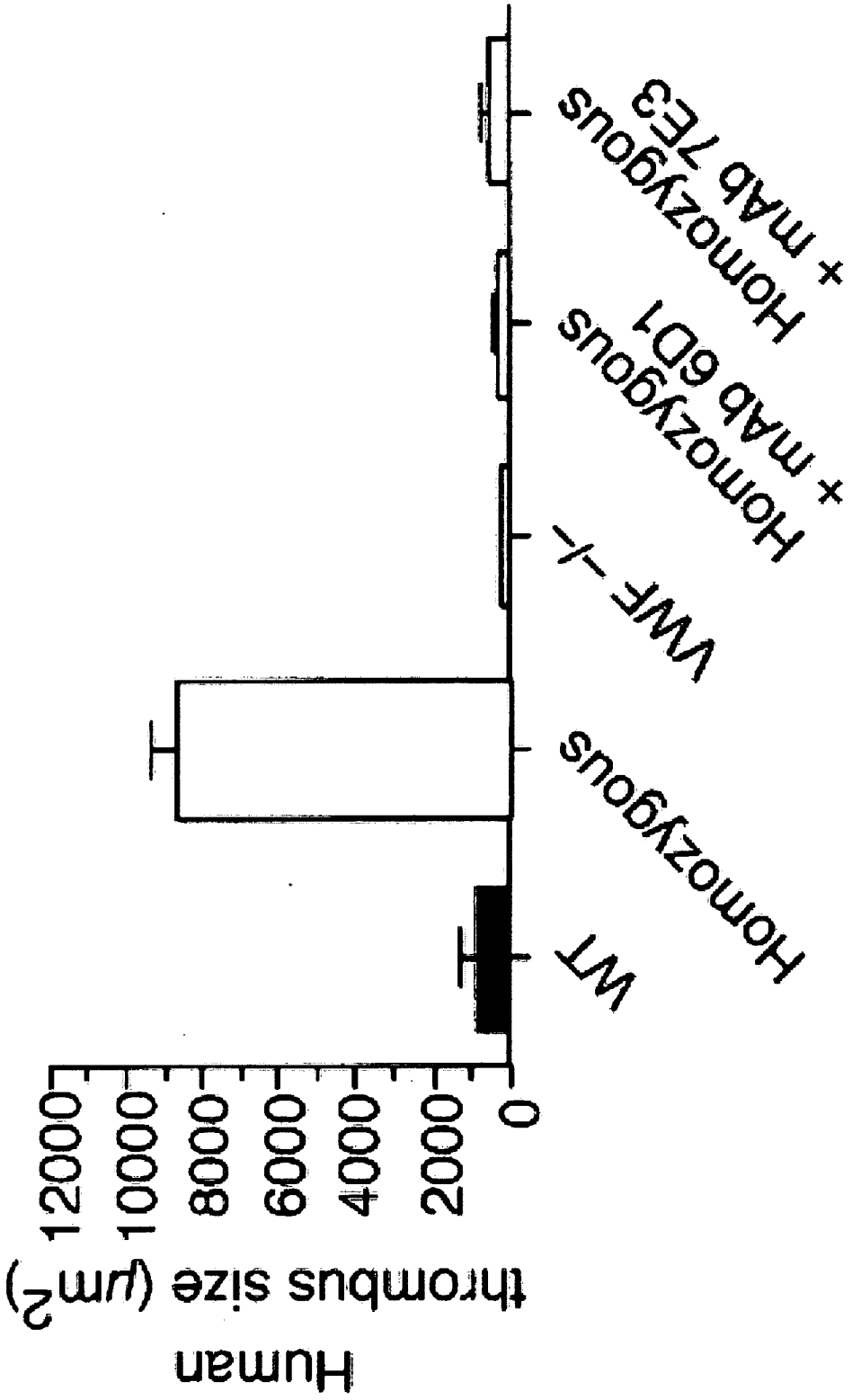

Human thrombus size (μm²)
12000
10000
8000
6000
4000
2000
0
WT
Homozygous
VWF -/-
Homozygous + mAb 6D1
Homozygous + mAb 7E3

FIG. 41E

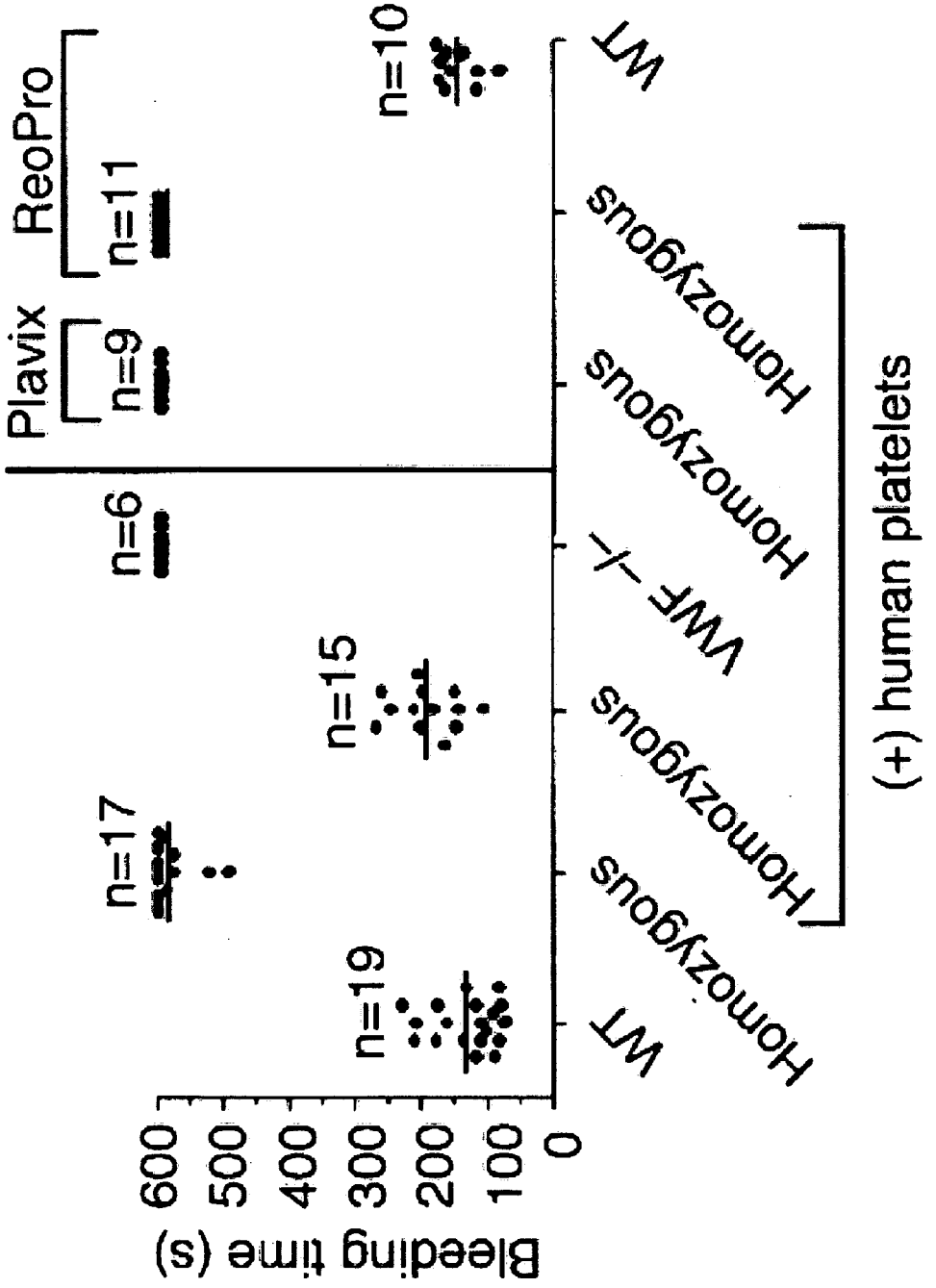

Bleeding time (s)
600
500
400
300
200
100
0
n=17
n=19
n=15
n=6
n=9
n=11
n=10
Plavix
ReoPro
WT
Homozygous
Homozygous
VWF -/-
Homozygous
WT
(+) human platelets

FIG. 41F

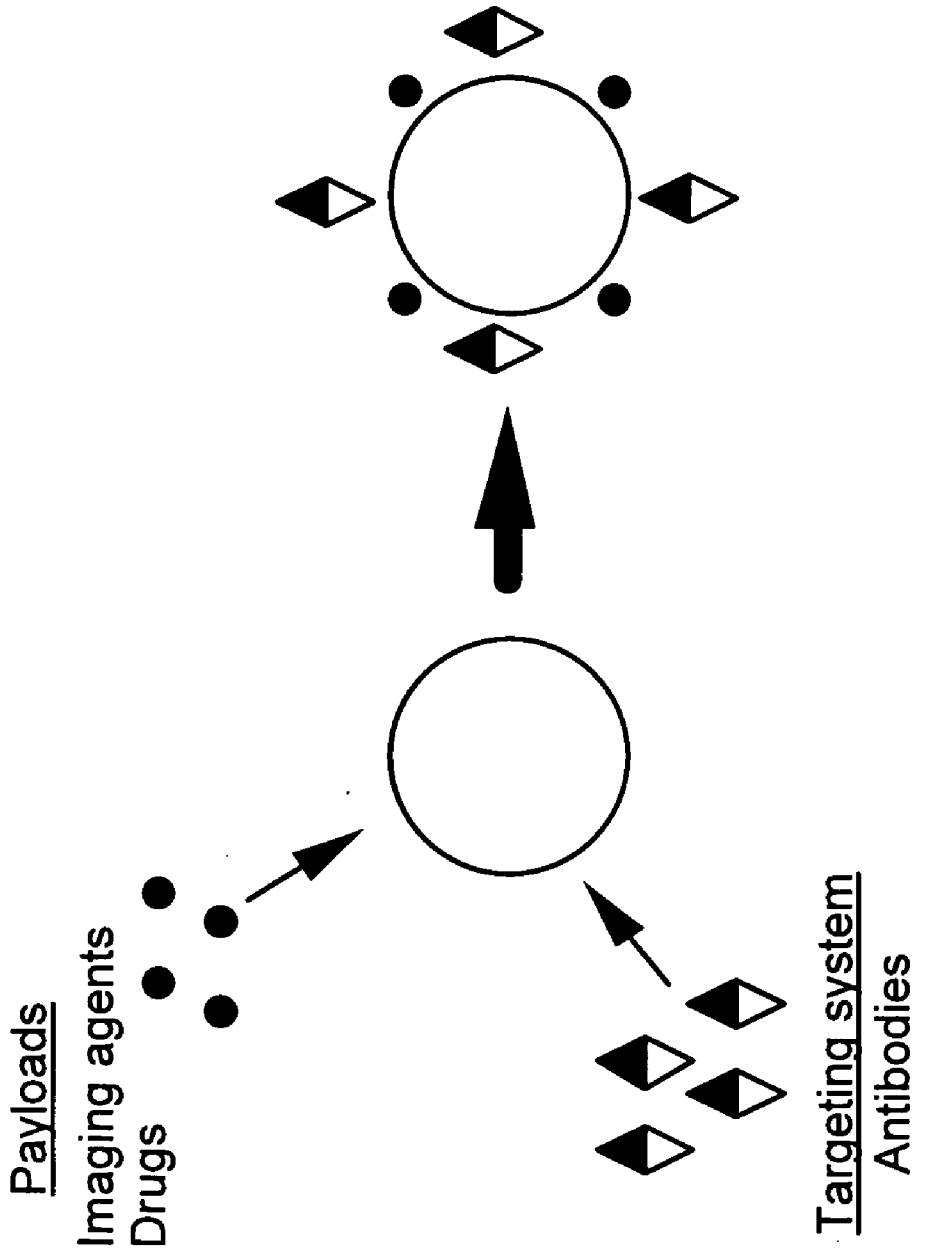

Payloads
Imaging agents
Drugs
Targeting system
Antibodies

FIG. 42

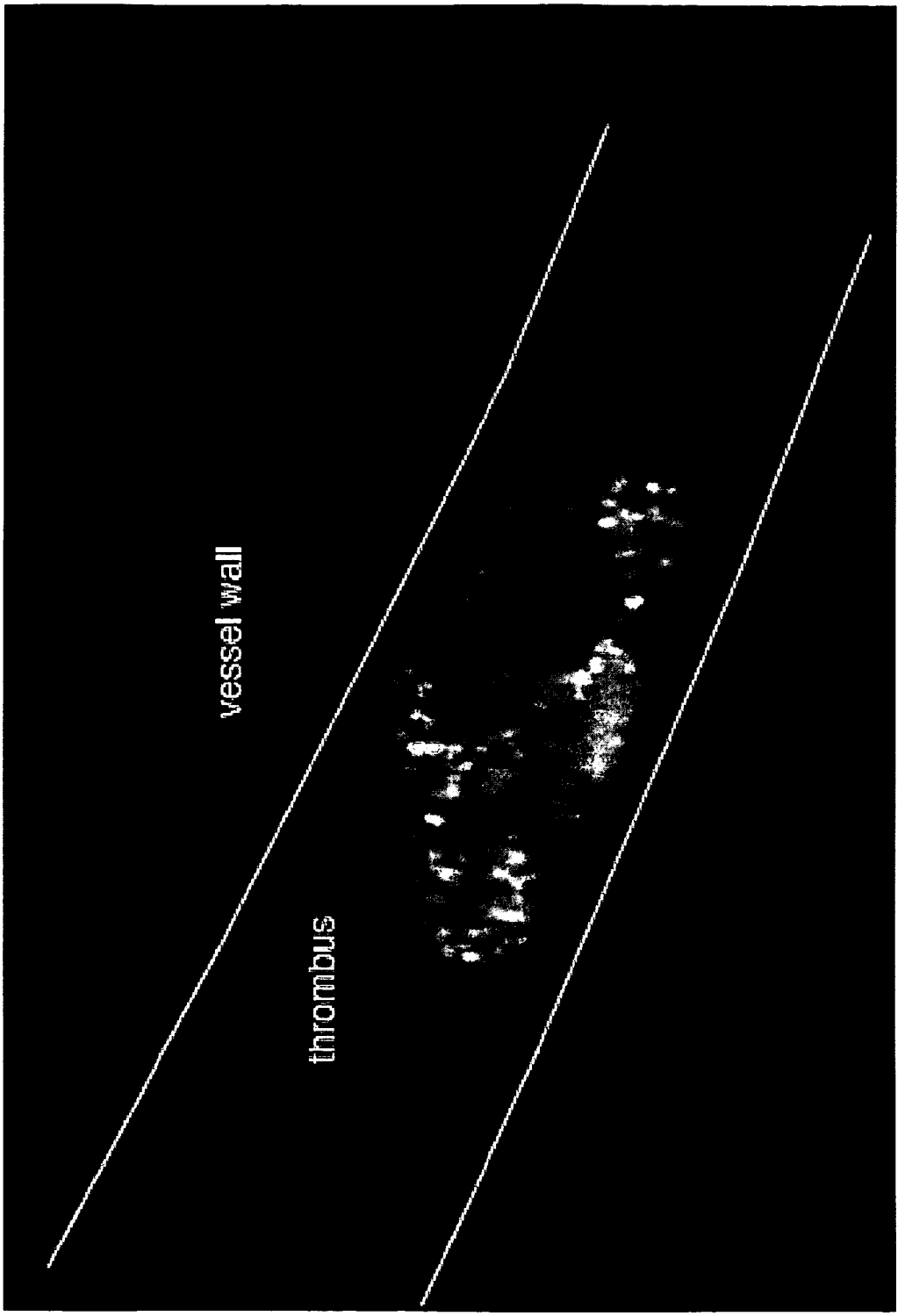


FIG. 43

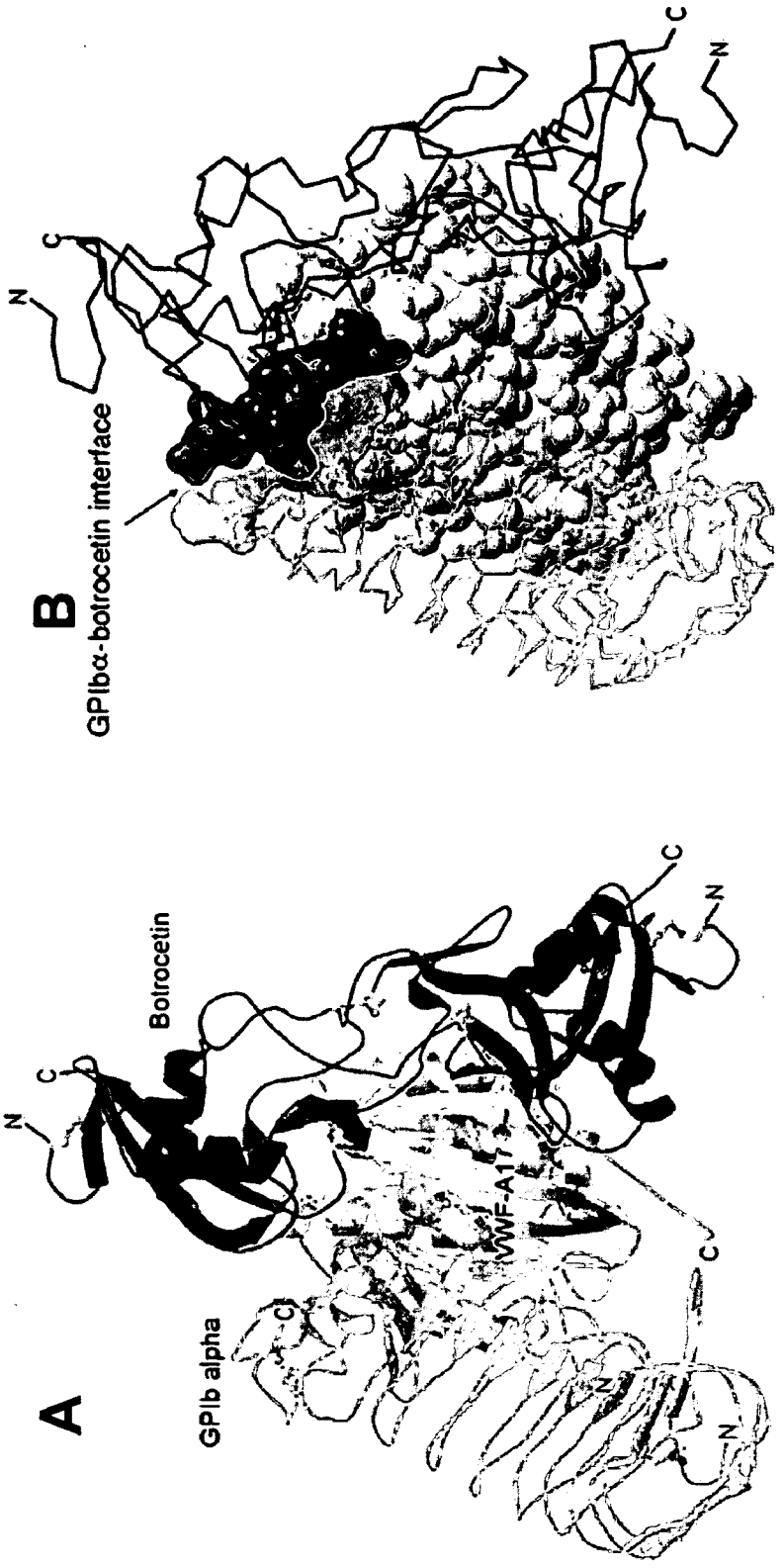

A
GPIb alpha
Botrocetin
VWF-A1
B
GPIbα-botrocetin interface
N
C

FIG. 44

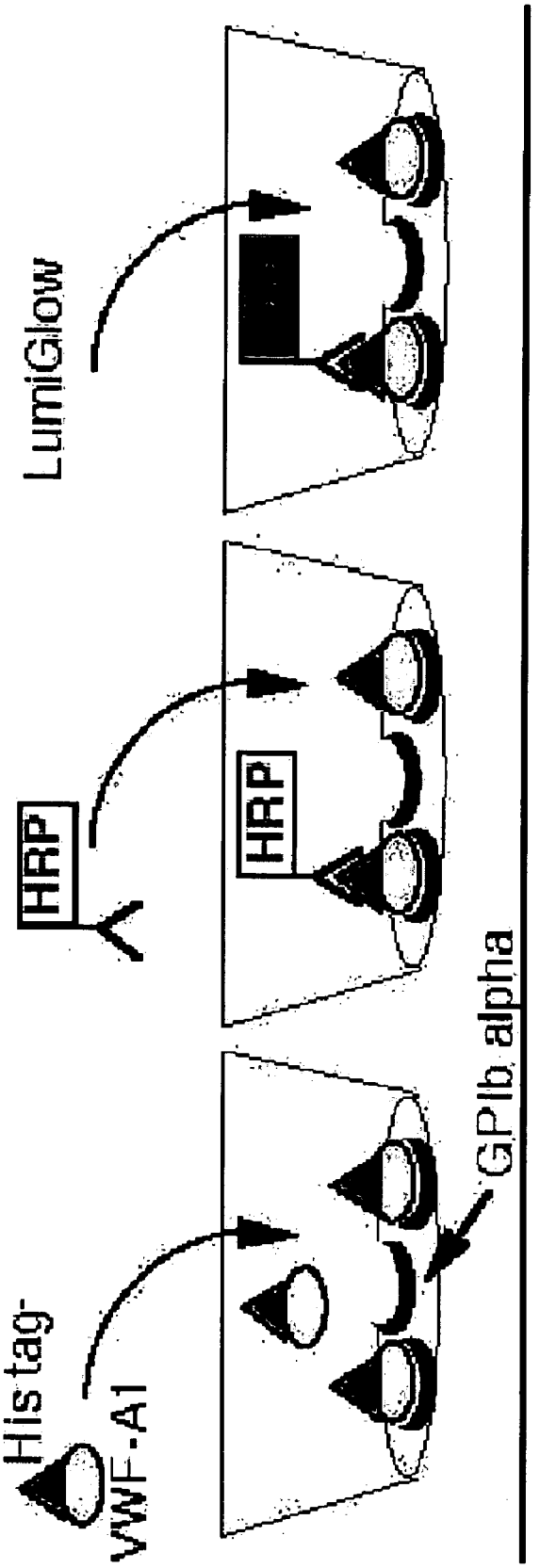


FIG. 45A

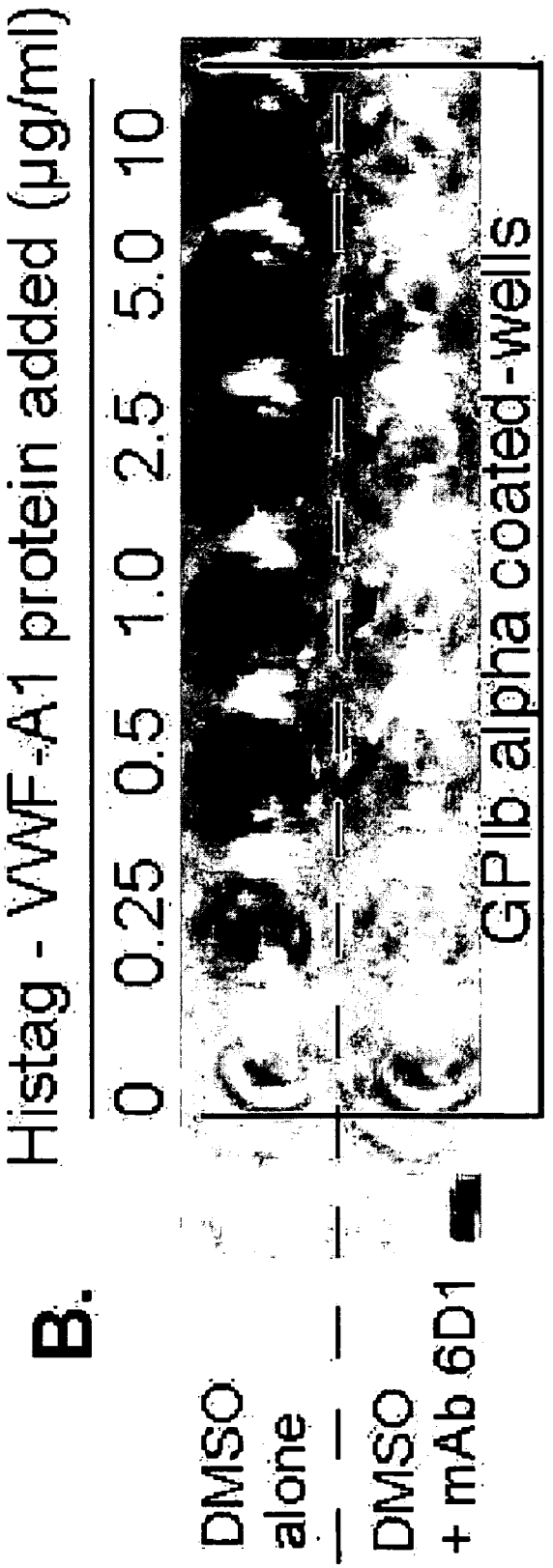

B.
Histag - VWF-A1 protein added (µg/ml)
0 0.25 0.5 1.0 2.5 5.0 10
GPIb alpha coated-wells
DMSO alone
DMSO + mAb 6D1

FIG. 45B

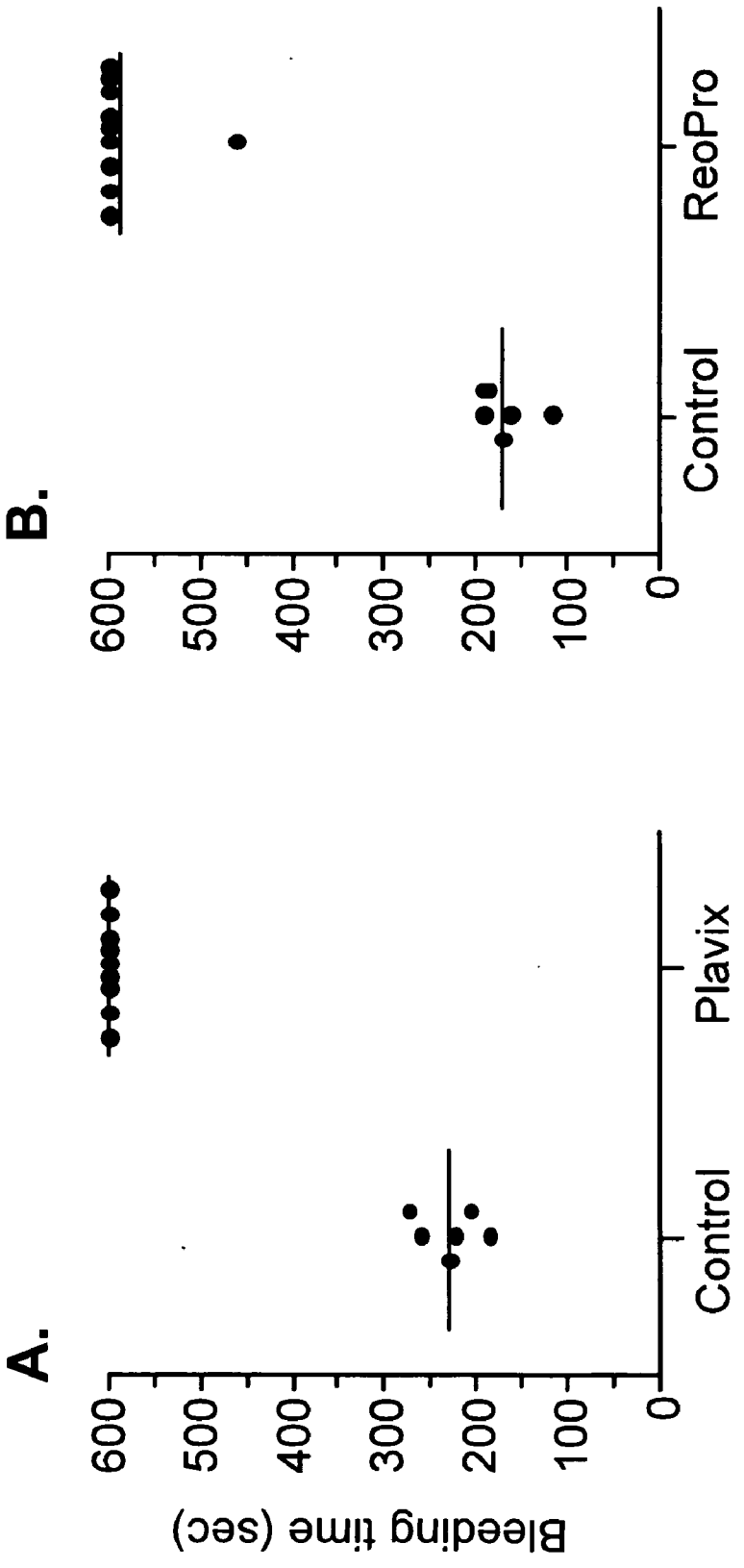

A.
B.
Bleeding time (sec)
600
500
400
300
200
100
0
Control
Plavix
Control
ReoPro

FIG. 46

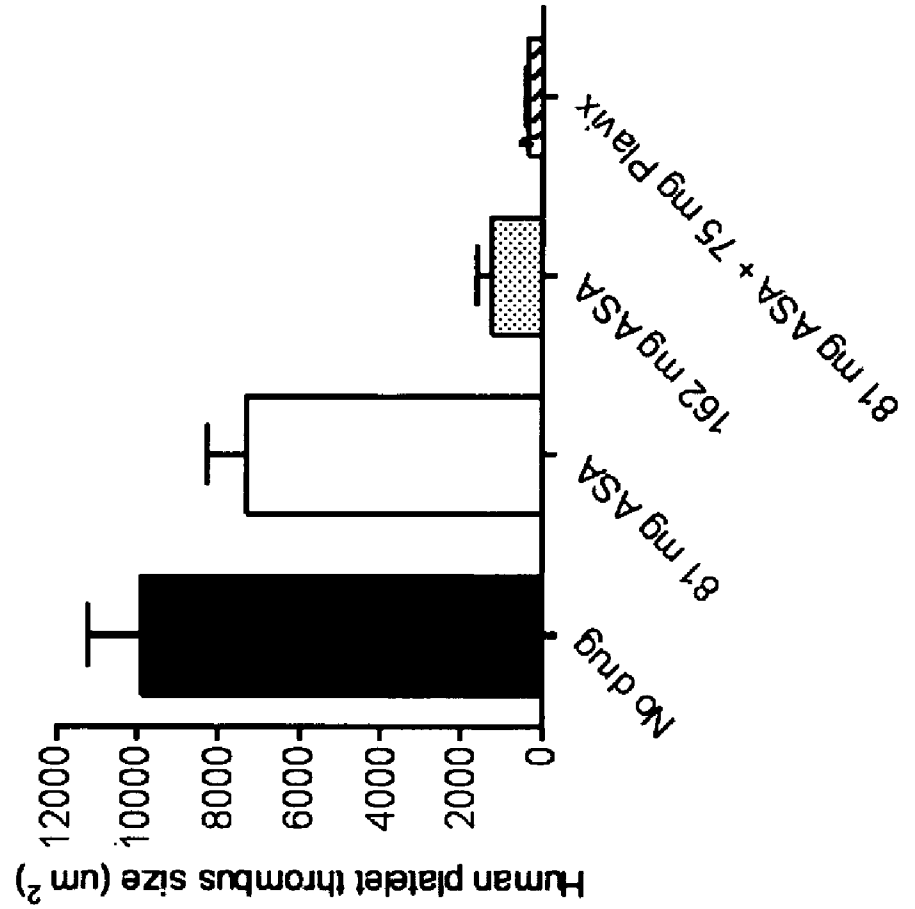

Human platelet thrombus size (um 2)
12000
10000
8000
6000
4000
2000
0
No drug
81 mg ASA
162 mg ASA
81 mg ASA + 75 mg Plavix

FIG. 47

METHODS FOR TESTING ANTI-THROMBOTIC AGENTS

[0001] This application is a continuation-in-part of International Application No. PCT/US2007/015043 filed on Jun. 28, 2007, which claims the benefit of priority of U.S. Ser. No. 60/817,600 filed on Jun. 29, 2006, the contents of which are hereby incorporated in their entirety.

GOVERNMENT INTERESTS

[0002] This invention was made with support from the U.S. Federal Government under Grant No. 5RO1HL63244-7 awarded by the National Heart, Lung, and Blood Institute (NHLBI). As such, the United States government has certain rights in this invention.

[0003] Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described and claimed herein.

BACKGROUND OF THE INVENTION

[0005] The ability of platelets to rapidly stick to the damaged wall of arterial blood vessels is critical for preventing blood loss (hemorrhage). Inappropriate deposition of these hemostatic cells in arterial blood vessels due to pathological disease processes such as atherosclerosis can result in lack of blood flow to vital organs such as the heart and brain. Thus a delicate balance exists between providing adequate hemostasis without causing blockage of blood vessels by excessive platelet deposition (a.k.a. thrombus formation).

[0006] von Willebrand Factor (VWF) is a multidomain, plasma glycoprotein of complex multimeric structure which is synthesized by vascular endothelial cells and megakaryocytes (1-3) (FIG. **1**A). Its presence in the blood is vital to maintaining the integrity of the vasculature. To accomplish this task, VWF forms a "bridge" between the injured vessel wall and platelets by virtue of its ability to interact with extracellular matrix components, such as collagen, and receptors expressed on platelets, such as glycoprotein Ib alpha (4-9). It also binds to and confers stability to factor VIII (10). The importance of this glycoprotein in hemostasis is underscored by the occurrence of clinical bleeding when the plasma VWF levels fall below 50 IU/dL (type I von Willebrand's disease, VWD), or when functional defects in the protein occur (type 2 VWD) (11,12).

[0007] Upon surface immobilization of VWF at sites of vascular injury, it is the role of the A1 domain of VWF (residues 1260-1480) to initiate the process of platelet deposition at sites of vascular injury and under conditions of high rates of shear flow (>1,000 s^{-1}; Ruggeri, Z. M. et al. *Blood.* 108, 1903-1910 (2006)). The critical nature of this interaction is exemplified by the bleeding disorder, termed type 2M VWD, which results from the incorporation of loss-of-function mutations within this domain that perturb interactions with GPIb alpha (Sadler, J. E. et al. (2006) *J. Thromb. Haemost.* 4, 2103-2114; Rabinowitz, I. et al. (1992) *Proc. Natl. Acad. Sci. USA* 89, 9846-9849; Cruz et al., (2000) *J. Biol. Chem.* 275, 19098-19105). In addition, recombinant VWF multimers lacking the A1 domain cannot support platelet adhesion at high rates of flow despite retaining the ability to interact with collagen (Sixma et al., (1991) *Eur J Biochem.* 196:369-75).

[0008] The structure of the A1 domain includes the α/β fold with a central β-sheet flanked by α-helices on each side as well as one intra-disulfide bond (Cys1272-Cys 1458), but no MIDAS motif (Emsley et al., (1998) *J Biol Chem.* 273:10396-401). Its overall shape is cuboid, with the top and bottom faces forming the major and minor binding sites, respectively, that interact with the concave surface of GPIbα. The most extensive contact site buries ~1700 $Å^2$ of surface area, interacting with LRR five to eight and the C-terminal flank of the GPIbα (Huizinga, E. G. et al. (2002) *Science* 297, 1176-1179). For this to occur, the β-switch region of this platelet receptor undergoes a conformation change so that it aligns itself with the central beta sheet of the A1 domain. The smaller site (~900 $Å^2$) accommodates the binding of the β-finger and the first LRR of GPIbα, an event that appears to require the displacement of the amino-terminal extension of the A1 domain. Based on these findings as well as the preferential localization of mutations in humans within this region, which enhance GPIbα binding, it is speculated that the amino-terminal extension regulates the adhesive properties of this domain. This is also supported by the fact that recombinant A1 proteins lacking this extension have a higher affinity for this platelet receptor (Sugimoto et al., (1993) *J Biol Chem.* 268:12185-92). Despite these observations, the physiological relevance of such structural changes in this receptor-ligand pair remains to be determined as well as the contribution of other domains to this process.

[0009] In addition to its role in hemostasis, VWF also contributes to pathological thrombus formation on the arterial side of the circulation. This may be the consequence of injury to the blood vessel wall from inflammatory disease states and/or medical/surgical interventions. Pathological thrombus formation is the leading cause of death in the Western world. Thus, pharmaceutical companies have committed considerable resources towards the research and design of drugs to prevent or treat thrombosis. However, there remains an urgent need to develop new and improved therapies such as those aimed at reducing platelet and/or VWF interactions with the injured arterial wall. One major hurdle hindering drug development in this field is the lack of an appropriate small animal model of thrombosis to test promising therapies. For instance, differences in the structure or isoform of protein receptors or ligands on mouse vs. human platelets that are critical for the activation and/or binding of these cells to the injured vessel wall preclude testing of drugs developed against human platelets in a mouse model of thrombosis. Moreover, this issue cannot be overcome by simply transfusing mice with human platelets as we have observed that mouse VWF does not support significant interactions with human cells (see below). Thus, the development of a "humanized" mouse model of hemostasis and thrombosis would potentially expedite drug discovery and testing.

[0010] That said, we have discovered that only one amino acid difference between mouse and human VWF-A1 domains accounts for most of the inability of the former to interact with human platelets and vice versa. With this knowledge in hand,

we have genetically altered a mouse to express VWF that contains this amino acid found in human VWF-A1, imparting on it the ability to support adhesion of human platelets to a level observed for its human counterpart. As a result, not only are we uniquely poised to better understand the molecular mechanisms governing human platelet binding at sites of vascular injury in vivo, but now have the capability to perform pre-clinical testing of anti-thrombotic agents and targeted molecular imaging agents directed against human platelet cells in a living animal. The material contained within this document describes the features of this unique biological platform for drug testing the testing of drugs and targeted molecular imaging agents.

BRIEF DESCRIPTION OF THE FIGURES

[0011] FIG. **1**A is a schematic representation of the prepro form of VWF. From top to bottom: repeated homologous regions; A1 and A3 disulfide loops and functional domains of the mature VWF subunit.

[0012] FIG. **1**B is an illustration depicting the sequential adhesive and activation events that promote platelet deposition at sites of vascular injury.

[0013] FIG. **2** is a model depicting the location of residues in the human VWF-A1 domain that if mutated, diminish GPIb alpha-mediated platelet binding in flow. Type 2M mutations are in red, residues identified by alanine scanning mutagenesis are in blue, and residues identified by crystal structure are in green.

[0014] FIG. **3**, panel A, is a structure model depicting residues associated with type 2M (red) or type 2B (yellow) VWD. Panel B represents a silver stained gel that clinically depicts a type 2B VWD disease state individual, which is characterized by a loss of circulating high molecular weight VWF multimers (HMWM, FIG. **3**B, Lane 2).

[0015] FIG. **4** shows structure models of the human VWF-A1 domain. FIG. **4**A represents the location of the Ile1309 mutation and its proposed effects on residues critical for GPIb binding. FIG. **4**B shows the loss of the isoleucine methyl group allows a water molecule to enter, which ultimately results in changes in orientation of the G1324 peptide plane and the side chain of H1326 as depicted, residues critical for GPIb binding.

[0016] FIG. **5** is a space-filling model of the botrocetin-A1 complex with sites involved in GPIb alpha binding and location of type 2B mutations indicated (panel A), wherein botrocetin does not alter the conformation of VWF-A1. In panel B, minor conformational changes in the A1 domain are represented. Uncomplexed (blue) and complexed (green) mutant domains are superimposed onto the WT structure (red).

[0017] FIG. **6** is schematic wherein the uncomplexed A1 domain, an amino-terminal extension (pink) appears to block a binding site for the amino-terminal β-hairpin (orange arrows) of GPIb alpha. Binding requires the amino-terminal extension of A1 to move, and also induces the β-switch (yellow loop) of GPIb alpha to form a β-strand motif.

[0018] FIG. **7** depicts microscope images wherein the use of platelets in lieu of recombinant proteins or transfected cells as the immobilized substrate enables evaluation of GPIb alpha in its native form (i.e. correct orientation and proper post-translational modification). Platelet coverage of <10% can be bound in this manner and can remain relatively unactivated for up to 30 min as evident by morphology on light microscopic examination (FIG. **7**A) and lack of expression of P-selectin by fluorescence microscopy (FIG. **7**B).

[0019] FIG. **8** represents quantitations of bead-platelet interactions under flow. FIGS. **8**A and **8**B demonstrates the direct visualization of bead-platelet interaction under flow (60×DIC microscopy). An approaching bead moving at a velocity of 609±97 um/sec (wall shear stress of 1.5 dyn cm^{-2}) is captured by a surface-immobilized platelet at (t=12.8 msec), pivots a distance of less than 3 μm in under 40 msec, and is then released after a pause time of t_p=228.2 msec into the flow stream (escape velocity=288+/−90.4 μm/sec). FIG. **8**C depicts representative experiments of k_{off} values for WT human VWF-A1 coated beads based on a distribution of interaction (pause) times. FIG. **8**D shows that the kinetics of the GPIb alpha tether bond are identical whether platelets are metabolically inactivated or fixed in paraformaldehyde to prevent activation upon surface-immobilization.

[0020] FIG. **9** represents the deduced single-letter amino acid sequence of mouse VWF-A1 domain (M VWF) compared to its human counterpart (H VWF) from amino acid 1260 to 1480. The locations of cysteines forming the loop structure are numbered (1238 and 1472) and differences in residues are highlighted in red. Conversion of the arginine (R) in the mouse A1 domain to histidine (H) as found in its human counterpart (blue χ) enables mouse VWF to bind human platelets.

[0021] FIG. **10** represents graphs of ristocetin-induced platelet aggregation assays (RIPA). Concentrations of the ristocetin modulator known to cause agglutination of human platelets (~1.0 mg/ml) had no effect using murine platelet rich plasma (FIG. **10**B, FIG. **10**D). Incubation of murine platelet rich plasma (PRP) with thrombin resulted in >90% platelet aggregation (FIG. **10**A). Concentrations of ≧2.5 mg/ml of modulator resulted in murine platelet aggregation (30%, FIG. **10**C).

[0022] FIG. **11** depicts a multimer gel analysis of purified VWF from human (lane 1, FIG. **11**A) and mouse (lane 2, FIG. **11**A) plasma. The ability of human and mouse VWF to mediate platelet adhesion in flow was determined in order to evaluate platelet interactions between human and murine VWF with GPIb alpha, as depicted in the bar graph of FIG. **11**B. Surface-immobilized murine VWF supports adhesion of syngeneic platelets (1×10^8/ml) at a shear rate encountered in the arterial circulation (1600 s^{-1}) as observed for the human plasma protein (FIG. **11**B, first panel). In contrast, murine VWF did not support significant interactions with human platelets and vice versa.

[0023] FIG. **12** is an image of a gel of mouse and human VWF-A1 highly purified protein, which was dialyzed against 25 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.8. SDS-PAGE analysis revealed a prominent protein band of 34,000 Da for mouse VWF-A1 under non-reducing conditions.

[0024] FIG. **13** depicts bar graphs of a series of in vitro flow chamber assays performed to assess platelet adhesion, wherein human or murine platelets (5×10^7/ml) were infused through a parallel plate flow chamber containing glass cover slips coated with either human (H) VWF-A1 or murine (M) VWF-A1 protein (100 μg/ml final concentration) at a shear rate of 800 s^{-1}. M VWF-A1 protein supported platelet adhesion as efficiently as its human counterpart under physiological flow conditions (FIG. **13**A). The translocation of mouse platelets occurred to a similar degree as its human counterpart under physiological flow conditions (FIG. **13**B). However, human platelets had a reduced capacity to interact with M

VWF-A1 protein and mouse platelets had a reduced capacity to interact with H VWF-A1 protein in flow.

[0025] FIG. **14**, panel A, represents purified bacterial His-tagged VWF-A1 protein and non-His tagged VWF-A1 protein that was analyzed by SDS-PAGE (12.5%) under non-reducing and reducing conditions. FIG. **14**B depicts a bar graph of a human platelet adhesion assay to recombinant VWF proteins with and without the presence of a His-tag at a shear rate of 800 s^{-1}.

[0026] FIG. **15** represents models of the crystal structure of VWF-A1 domains solved using a recombinant protein. The main chain schematic of the mouse VWF-A1 domain, with β-strands (arrows) and helices (coils), is shown in FIG. **15**A. The two cysteines involved in the disulfide bridge are shown as yellow spheres. FIG. **15**B demonstrates that the C-alpha atoms of human (red) and mouse (blue) VWF-A1 domains closely overlap. FIG. **15**C shows the model of the murine VWF-A 1 domain and the residues that purportedly interact with GPIb alpha, wherein amino acid residue 1326 of mouse (M) VWF-A1 was mutated to the corresponding amino acid at the identical location in its human counterpart (from Arg to His).

[0027] FIG. **16** shows graphs that depict platelet adhesion assays (FIG. **16**A) and platelet translocation measurements (FIG. **16**B). The ability of murine and human platelets to interact with a mutant protein substrate (human VWF-A1 domain wherein amino acid residue 1326 was mutated from His to Arg and mouse VWF-A1 domain wherein amino acid residue 1326 was mutated from Arg to His) was evaluated at a wall shear rate of 800 s^{-1}.

[0028] FIG. **17** represents data from an ELISA assay. Following several injections of mouse (M) VWF-A1, serum was collected from rats and screened by ELISA for anti-VWF-A1 antibodies. Spleens from animals with the highest antibody titers were harvested and splenocytes fused with Sp2/0 mouse myeloma cells (54). Supernatants of hybridomas were screened for reactivity to murine (M) VWF-A1 by ELISA. Pre-immune rat serum was used as control. Mabs to M VWF-A1 not only reacted with WT and mutant proteins (1324G>S) but also recognized native VWF purified from mouse plasma.

[0029] FIG. **18** shows representative graphs depicting the distribution of interaction times for more than 35 individual transient attachment events at various times. Analysis of the distribution of interactions times between human or murine VWF-A1 coated beads and their respective platelet substrates, as measured by high temporal resolution video microscopy, indicate that >95% of all transient tether bonds events fit a straight line, the regressed slope of which corresponded to a single k_{off}, wherein the cellular off-rates of these quantal units of adhesion for the wild type human (H) and murine (M) proteins are found in FIGS. **18**A and B and M VWF-A1 protein containing the type 2B mutation I1309V (1309I>V) corresponds to FIG. **18**C.

[0030] FIG. **19** shows graphs that represent an assessment of transient tether events (FIG. **19**A) and analysis of the distribution of interactions times (FIG. **19**B) between human VWF-A1 coated microspheres and human immobilized platelets. The type 2B mutation Ile1309 Val (1309I>V) was incorporated into recombinant human (H) VWF-A1 containing either the type 2M mutation Gly1324Ser (1324G>S) or the function reducing mutation His1326Arg (1326H>R).

[0031] FIG. **20** is a scheme for generating transgenic mice with mutant VWF-A1 domains. FIG. **20**A is a diagram of a knock-in construct for proposed mutations in the VWF-A1 domain of mice. FIG. **20**B represents Southern blot hybridization with probe "a" or "b", respectively, to determine if the construct was appropriately targeted.

[0032] FIG. **21** represents Southern blot analysis wherein heterozygous and homozygous mice for the amino acid substitution at residue 1326 (R1326H; 1326R>H) display the Arg1326His mutation (lanes 2 and 3 respectively) while wild-type animals did not (lane 1).

[0033] FIG. **22** represents sequence analysis of purified PCR products of WT, heterozygous, or homozygous VWF-A1 domains wherein the red-boxed area denotes the conversion of Arg to His (CGT in FIG. **22**A wherein the codon corresponds to Arg and CAT in FIG. **22**C wherein the codon corresponds to the amino acid His).

[0034] FIG. **23** is a graph of an ELISA assay which demonstrated that conversion of Arg to His in the mouse A1 domain did not alter plasma protein levels of VWF in mutant mice nor its ability to form multimers. The ELISA assay detected mouse VWF in plasma obtained from WT and homozygous (KI) animals, but not from plasma obtained from animals deficient in VWF (VWF KO).

[0035] FIG. **24** is a gel image of multimer gel analysis of plasma VWF that revealed an identical banding pattern between mouse and human VWF. Incorporation of His at position 1326 in the mouse A1 domain had no effect on multimerization of VWF in mutant mice.

[0036] FIG. **25** is a graphical representation of the bleeding times (s) observed in the mutant VWF-A1 mice that are either heterozygous or homozygous for the 1326R>H mutation. Results are compared to normal counterparts and VWF-deficient mice. Tail cut=1 cm.

[0037] FIG. **26** is a bar graph depicting thrombus formation induced by perfusion of whole blood from either wild type (WT) or homozygous mutant mice over surface-immobilized collagen in vitro wherein an 80% reduction in thrombus formation was observed compared to WT controls.

[0038] FIG. **27** are micrographs that demonstrate reduced thrombus formation occurring when whole blood from either the knock-in animals (homozygous for the R1326H mutation) or WT is perfused over collagen-coated cover slips at a shear rate of 1600 s^{-1} indicating a 70% reduction in thrombi formed on collagen as compared to WT controls.

[0039] FIG. **28** demonstrates a platelet adhesion assay in flow. R1326H mutant mouse VWF promotes interactions with human platelets under physiologic flow conditions, wherein anticoagulated human blood was infused over surface-immobilized WT or mutant mouse plasma VWF at 1600 s^{-1} as shown in the micrographs of FIG. **28**A. FIG. **28**B is a graph that depicts the amount of human platelets that bound to WT murine VWF or R1326H mutant murine VWF.

[0040] FIG. **29** are transmitted light micrographs demonstrating that homozygous mutant mice infused with human (FIG. **29**A) but not mouse platelets (FIG. **29**B) were able to generate an arterial thrombus that occludes the vessel lumen in response to laser-induced vascular injury as depicted by intravital microscopy.

[0041] FIG. **30** is a bar graph that depicts the average bleeding time for mice receiving blood-banked human platelets (~3 minutes for a 1 cm tail cut) or given an intravenous infusion of a physiological buffered saline solution (10 minutes (end point)).

[0042] FIG. **31** is a schematic representing the isolation of the cremaster muscle and the catheter set-up used in intravital microscopy assays to assess thrombus formation.

[0043] FIG. **32** is a schematic of an intravital microscopy method.

[0044] FIG. **33** demonstrates images of mouse platelet interactions and a bar graph of such interactions in a wild type animal. FIG. **33**A are representative intravital photomicrographs that depict the range of platelet interactions that occur at a site of vascular injury (60×). Platelets were observed to either transiently pause (*) or rapidly tether to and translocate (TP) on damaged arterial endothelium. A composite image demonstrates translocation of two platelets over a 3 s interval of time (panel 6). FIG. **33**B depicts interacting platelets at the site of arterial injury that were classified as either undergoing translocation or firm adhesion (sticking) during an observation period of 1 min.

[0045] FIG. **34** represents photomicrographs that depict the vessel wall in a wild type mouse in the (A) absence of injury or (B) post-laser-induced injury as visualized under transillumination (40× objective). Thrombus is indicated by the arrows.

[0046] FIG. **35** is a graphical representation of the bleeding phenotype observed in the mutant VWF-A1 1326R>H heterozygous or homozygous mouse compared to its WT counterpart when tails were cut either 5 mm (FIG. **35**A) or 15 mm (FIG. **35**B) from the tip of the tail.

[0047] FIGS. **36**A-B are graphs that depict ex vivo analysis of human platelet interactions with plasma VWF or recombinant VWF-A1 proteins. Accumulation of human platelets on surface-immobilized plasma human or mouse VWF (FIG. **36**A) or recombinant human or mouse A1 domain proteins (FIG. **36**B) after 4 min of perfusion with whole blood (shear rate of 1600 s^{-1}) is shown. Data are representative of three separate experiments performed in triplicate (mean±s.e.m.).

[0048] FIGS. **36**C-D are graphs that depict ex vivo analysis of mouse platelet interactions with plasma VWF or recombinant VWF-A1 proteins. Accumulation of murine platelets on surface-immobilized human or mouse plasma VWF (FIG. **36**C) or recombinant human or mouse A1 domain proteins (FIG. **36**D) after 4 min of perfusion with whole blood (shear rate of 1600 s^{-1}) is shown. Data are representative of three separate experiments performed in triplicate (mean±s.e.m.).

[0049] FIGS. **37**A-B are structural representations of human and murine VWF-A1 domains. FIG. **37**A depicts the alignment of Cα atoms for human (blue) and murine (red) A1 domains. Key residues described in EXAMPLE 4 are shown as red spheres or as ball-and stick side-chains. FIG. **37**B is a 90° rotation about a horizontal axis to reveal the packing of residue 1397 (Phe in human, Leu in mouse) that results in a 3 Å shift (blue arrow) of helix α4.

[0050] FIGS. **37**C-D are structural representations of human and murine GPIbα-VWF-A1 complexes. FIG. **37**C depicts the model of the murine-murine complex. FIG. **37**D depicts the crystal structure of the human-human complex. Salt bridges are circled and key residue differences are boxed. Zooms reveal details of the electrostatic interactions at the β-switch contact region. The region of contact involving helix α3 of the A1 domain and one face of the LRR repeats of GPIbα is highly conserved between species, except for two residue changes that do not participate in bond formation: GPIbα E151K and VWF-A1 G1370S (human:mouse). Thus, minor differences in this region are unlikely to contribute to a reduction in binding between the murine and human proteins. This is also the case with the contact area located at the bottom of the A1 domain, which is invariant in both species and participates in salt-bridge formation (red circle).

[0051] FIG. **37**E is a model of the human GPIbα-murine A1 complex, showing the loss (green arrow) and gain (blue circle) of salt-bridges. The upper zoom shows the interspecies interface at the β-switch region, revealing the electrostatic clash. The lower zoom shows the murine VWF-A1 point mutant 1326R>H, which removes the electrostatic clash and now closely resembles the human-human complex.

[0052] FIG. **37**F is a model of the murine GPIbα-human VWF-A1 complex. Two salt-bridges are lost as compared to the murine complex; murine GPIbα D238 with residue 1326 due to the R>H change in human VWF-A1, and murine GPIbα K237 with residue 1330 owing to the E>G change in the human protein. Moreover, neither the chimeric nor murine complex forms a salt-bridge between residues 225 and 1395 on GPIbα and VWF-A1, respectively, as compared to its human counterpart (green circle). The upper zoom shows the interspecies interface at the β-switch region; there is no electrostatic clash but no salt-bridge can form with H1326. The lower zoom shows the human point mutant 1326H>R, which adds a salt-bridge and now closely resembles the murine-murine complex.

[0053] FIG. **37**G is a graph that shows the accumulation of human platelets on surface-immobilized recombinant WT murine VWF-A1 domain proteins, those containing the selected mutations 1326R>H, 1330E>G and 1370S>G, or WT human VWF-A1 protein (shear rate of 1600 s^{-1}). Data are representative of three separate experiments performed in triplicate (mean±s.e.m.).

[0054] FIG. **38**A is schematic for the generation of the $VWF^{1326R>H}$ mouse that represents the targeting strategy for insertion of exon 28 containing adenine in lieu of guanine at position 3977 of the cDNA for murine VWF. R1, EcoRI; RV; EcoRV; B, BamHI; X, XhoI; pGK-TK, pGK-Neo, thymidine kinase/neomycin resistance cassette; ◀, loxP sites.

[0055] FIG. **38**B is a blot of a Southern analysis of tailed DNA digested with EcoR1. Wild-type (WT) allele, 14 kb; mutant allele, 6 kb using Probe A. FIG. **38**C represents the DNA sequencing of the tailed DNA demonstrating successful incorporation of adenine at position 3977 in heterozygous and homozygous animals (CGT>CAT). Sequence analysis of genomic DNA from these animals, 2 kb upstream and 6 kb downstream of exon 28, did not reveal any other alterations in nucleotide base pairs that would affect VWF production and/or function.

[0056] FIGS. **39**A-B represent the analysis of VWF gene transcription and translation. FIG. **39**A is a gel of RT-PCR of lung tissue from WT or mutant VWF mice to detect for A1, A2, and/or A3 domain message. β-actin analyzed to demonstrate use of equivalent amounts of mRNA. FIG. **39**B is a graph demonstrating VWF antigen levels in plasma obtained from WT littermates (pooled) or six individual mice homozygous for 1326R>H mutation as detected by ELISA. Data are representative of two independent experiments performed in triplicate.

[0057] FIG. **39**C is a gel showing the analysis of VWF multimers in plasma from WT or homozygous mutant animals. Normal human plasma as well as that obtained from a patient with type 2B VWD is shown for comparison.

[0058] FIG. **40** depicts representative photomicrographs showing murine platelet accumulation at sites of laser-induced arteriolar injury in WT or homozygous mutant animals 20 s and 2 min post-injury. White lines demarcate the extent of the thrombus.

[0059] FIG. **41**A is a graphical representation of the tail bleeding times (s) for heterozygous and homozygous $VWF^{1326R>H}$ and WT mice when tails were cut 1 cm from the tip of the tail. Each point represents one individual mouse and experiments were performed on five separate days.

[0060] FIG. **41**B is an ex vivo analysis of human platelet interactions with surface-immobilized plasma $VWF^{1326R>H}$ at a shear rate of 1,600 s^{-1}. A role for GPIb alpha on human platelets is demonstrated by the function-blocking antibody to this platelet receptor (mAb 6D1) to prevent adhesion in flow.

[0061] FIG. **41**C are microscopy images of in vivo analysis of human platelet interactions with murine plasma $VWF^{1326R>H}$ using infused fluorescently labeled human platelets into the vasculature of the cremaster muscle of mice. Human platelet accumulation was examined at sites of laser-induced arteriolar injury in WT (n=10) or homozygous mutant animals (n=12) using 2 channel confocal microscopy with excitation wavelengths of 488 nm (BCECF) and 561 nm (rhodamine 6G). Representative composite images of fluorescent images depicting human thrombus formation in homozygous mutant (upper panels) or WT (lower panels) mice (V=venule; A=arteriole). Rhodamine and BCECF are depicted in red and green, respectively, and merged is presented in yellow.

[0062] FIG. **41**D is a bar graph depicting the composition of thrombi (% of total area) in WT or homozygous mutant animals.

[0063] FIG. **41**E is a bar graph measuring thrombus size during an in vivo study of human platelet interactions with plasma $VWF^{1326R>H}$ to determine the effect of GPIbα or αIIbβ3 blockade on human platelet adhesion in vivo. The requirement for GPIb alpha-mediated adhesion is shown by the ability of a function-blocking antibody (mAB 6D1 or mAb 7E3) to GPIb alpha to prevent human platelet thrombus formation in vivo. Fluorescently labeled human platelet accumulation was examined at sites of laser-induced arteriolar injury in WT (n=6) or homozygous mutant animals (n=8). Data represent the mean±s.e.m.

[0064] FIG. **41**F is a graphical representation of tail bleeding times (s) for homozygous $VWF^{1326R>H}$ that received an infusion of either normal saline or human platelets prior to severing 10 mm of distal tail, wherein the ability of human platelets to restore hemostasis in homozygous $VWF^{1326R>H}$ and the effect of PLAVIX or ReoPro® on this process was examined. Each point represents one individual mouse and experiments were performed on 3 separate days.

[0065] FIG. **42** is a schematic depicting a perfluorocarbon nanoparticle capable of incorporating imaging agents (Gd^{+3}, ^{99m}Tc) and chemotherapeutics into the outer layers. Antibodies complexed to the surface of the particle can target the agent to specific sites within the body.

[0066] FIG. **43** is a photographic image depicting the accumulation of fluorescent PNP, coupled to an antibody that recognizes human alphaIIb beta 3 on the surface of human platelets, at a site of vascular injury in homozygous 1326R>H mutant mice infused with human platelets.

[0067] FIG. **44** is a graphical representation of the structure of the VWF-A1-GPIb alpha-botrocetin ternary complex. FIG. **44**A is a ribbon representation; GPIb alpha, green; botrocetin, red; A1, cyan. FIG. **44**B demonstrates the location of the previously unknown interface between GPIb alpha and botrocetin.

[0068] FIG. **45**A is a schematic representation showing that Recombinant GPIb alpha is surface-immobilized in a 96 well format. After blocking potential non-specific binding sites, recombinant VWF-A1 containing a His tag is added to the wells and allowed to interact with GPIb alpha for a specified period of time. The unbound material is removed by washing the wells and the complex formed between the 2 proteins detected by the addition of a HRP-conjugated antibody that binds to the His tag on A1. The amount of bound A1 can then be quantified by either fluorescence (addition of LumiGlow) or by color change.

[0069] FIG. **45**B is an image representing that the specificity of the interaction can be determined by the addition of the GPIb function blocking antibody 6D1 prior to the addition of recombinant VWF-A1. DMSO (0.5%) was added to illustrate that this reagent does not interfere with the assay.

[0070] FIG. **46** are graphs depicting the effect of Plavix (FIG. **46**A) or ReoPro (FIG. **46**B) on human platelet-induced hemostasis in homozygous $VWF^{1326R>H}$ mice.

[0071] FIG. **47** is a graph showing the efficacy of anti-platelet drugs administered to patients by studying the ability of platelets harvested from patients on therapies in the $VWF^{1326R>H}$ mouse.

SUMMARY OF THE INVENTION

[0072] The invention provides for a mouse model for pre-clinical screening and testing of candidate compounds, wherein the model comprises a mouse expressing plasma von Willebrand Factor protein that contains a mutation or combination of mutations in its A1 domain that change the mouse protein's binding specificity from being specific for mouse platelets to being specific for human platelets. Thus, the mutant A1 domain contained within mouse plasma VWF particularly supports the binding of human platelets in vivo and ex-vivo. In one embodiment, the mouse model is infused with human platelets. In another embodiment, human platelets are labeled ex-vivo or in vivo so as to be detected while in the animal.

[0073] The invention provides for where the mutation in the A1 domain of mouse VWF comprises 1263P>S, 1269N>D, 1274K>R, 1287M>R, 1302G>D, 1308H>R, 1313R>W, 1314I>V, 1326R>H, 1329L>1,1330E>G, 1333A>D, 1344T>A, 1347I>V, 1350T>A, 1370G>S, 1379H>R, 1381T>A, 1385T>M 1391P>Q, 1394A>S, 1397L>F, 1421S>N, 1439L>V, 1442G>S, 1449R>Q, 1466A>P, 1469Q>L, 1472Q>H, 1473V>M, 1475H>Q, 1479S>G, and any combination thereof, wherein the mutation corresponds to an amino acid position of human von Willebrand Factor A1 protein shown in SEQ ID NO: 6. In addition, the mouse VWF A1 domain can be fully or partially replaced with the human VWF A1 domain. In one embodiment, the mutation in mouse VWF-A1 is 1326R>H. In another embodiment, the mouse model comprises a device within a vessel, such as a stent or a graft, or mechanical, chemical, or heat-induced disruption of vascular endothelium in vivo. This model system is useful for testing compounds in an in vivo environment. The compounds can be tested for an effect on the interaction between human platelets and human-like (the mutant VWF-A1, 1326R>H, for example), or the actual human VWF-A1 domain. For example, the animal model can be used for pre-clinical testing of drugs in order to determine whether 1) there is a desired effect on hemostasis and/or thrombus formation or anti-thrombotic effect by the test drug or 2) there is an undesired effect on hemostasis and/or thrombus formation

or anti-thrombotic effect by a test drug not specifically designed to alter hemostasis and/or thrombus formation. In the latter case, many drugs are only identified as having an effect on clotting or bleeding once they are in human clinical trials, this animal model will fill an unmet need, which is to test such effects prior to clinical trials. The invention also permits testing of compounds targeted to the VWF-A1 domain that can correct the bleeding phenotype associated with a loss-of-function mutations (1326R>H, for example) by altering the kinetics of the interaction between GPIbα and VWF-A1 (for example, enhancing the on-rate and/or prolonging the bond lifetime as shown for the snake venom protein botrocetin.

[0074] The invention also provides for an isolated mutant human von Willebrand Factor A1 protein comprising one or more mutations selected from the group consisting of: 1263S>P, 1269D>N, 1274R>K, 1287R>M, 1302D>G, 1308R>H, 1313W>R, 1314V>I, 1326H>R, 1329I>L, 1330G>E, 1333D>A, 1344A>T, 1347V>I, 1350A>T, 1370S>G, 1379R>H, 1381A>T, 1385M>T, 1391Q>P, 1394S>A, 1397F>L, 1421N>S, 1439V>L, 1442S>G, 1449Q>R, 1466P>A, 1469L>Q, 1472H>Q, 1473M>V, 1475Q>H, 1479G>S, wherein each amino acid position corresponds to a position in SEQ ID NO: 6. The invention also provides for an isolated mutant human von Willebrand Factor A1 protein consisting of a 1263S>P mutation in an amino acid sequence of SEQ ID NO: 1. The invention also provides for an isolated mutant human von Willebrand Factor A1 protein consisting of a 1269D>N mutation in an amino acid sequence of SEQ ID NO: 1. The invention also provides for an isolated mutant human von Willebrand Factor A1 protein consisting of a 1274R>K mutation in an amino acid sequence of SEQ ID NO: 1. The invention also provides for an isolated mutant human von Willebrand Factor A1 protein consisting of a 1287R>M mutation in an amino acid sequence of SEQ ID NO: 1. The invention also provides for an isolated mutant human von Willebrand Factor A1 protein consisting of a 1302D>G mutation in an amino acid sequence of SEQ ID NO: 1. The invention also provides for an isolated mutant human von Willebrand Factor A1 protein consisting of a 1308R>H mutation in an amino acid sequence of SEQ ID NO: 1. The invention also provides for an isolated mutant human von Willebrand Factor A1 protein consisting of a 1313W>R mutation in an amino acid sequence of SEQ ID NO: 1. The invention also provides for an isolated mutant human von Willebrand Factor A1 protein consisting of a 1314V>I mutation in an amino acid sequence of SEQ ID NO: 1. The invention also provides for an isolated mutant human von Willebrand Factor A1 protein consisting of a 1326H>R mutation in an amino acid sequence of SEQ ID NO: 1. The invention also provides for an isolated mutant human von Willebrand Factor A1 protein consisting of a 1329I>L mutation in an amino acid sequence of SEQ ID NO: 1. The invention also provides for an isolated mutant human von Willebrand Factor A1 protein consisting of a 1330G>E mutation in an amino acid sequence of SEQ ID NO:1. The invention also provides for an isolated mutant human von Willebrand Factor A1 protein consisting of a 1333D>A mutation in an amino acid sequence of SEQ ID NO: 1. The invention also provides for an isolated mutant human von Willebrand Factor A1 protein consisting of a 1344A>T mutation in an amino acid sequence of SEQ ID NO: 1. The invention also provides for an isolated mutant human von Willebrand Factor A1 protein consisting of a 1347V>I mutation in an amino acid sequence of SEQ ID NO: 1. The invention also provides for an isolated mutant human von Willebrand Factor A1 protein consisting of a 1350A>T mutation in an amino acid sequence of SEQ ID NO: 1. The invention also provides for an isolated mutant human von Willebrand Factor A1 protein consisting of a 1370S>G mutation in an amino acid sequence of SEQ ID NO: 1. The invention also provides for an isolated mutant human von Willebrand Factor A1 protein consisting of a 1379R>H mutation in an amino acid sequence of SEQ ID NO: 1. The invention also provides for an isolated mutant human von Willebrand Factor A1 protein consisting of a 1381 A>T mutation in an amino acid sequence of SEQ ID NO:1. The invention also provides for an isolated mutant human von Willebrand Factor A1 protein consisting of a 1385M>T mutation in an amino acid sequence of SEQ ID NO: 1. The invention also provides for an isolated mutant human von Willebrand Factor A1 protein consisting of a 1391Q>P mutation in an amino acid sequence of SEQ ID NO: 1. The invention also provides for an isolated mutant human von Willebrand Factor A1 protein consisting of a 1394S>A mutation in an amino acid sequence of SEQ ID NO: 1. The invention also provides for an isolated mutant human von Willebrand Factor A1 protein consisting of a 1397F>L mutation in an amino acid sequence of SEQ ID NO: 1. The invention also provides for an isolated mutant human von Willebrand Factor A1 protein consisting of a 1421N>S mutation in an amino acid sequence of SEQ ID NO: 1. The invention also provides for an isolated mutant human von Willebrand Factor A1 protein consisting of a 1439V>L mutation in an amino acid sequence of SEQ ID NO: 1. The invention also provides for an isolated mutant human von Willebrand Factor A1 protein consisting of a 1442S>G mutation in an amino acid sequence of SEQ ID NO: 1. The invention also provides for an isolated mutant human von Willebrand Factor A1 protein consisting of a 1449Q>R mutation in an amino acid sequence of SEQ ID NO: 1. The invention also provides for an isolated mutant human von Willebrand Factor A1 protein consisting of a 1466P>A mutation in an amino acid sequence of SEQ ID NO: 1. The invention also provides for an isolated mutant human von Willebrand Factor A1 protein consisting of a 1469L>Q mutation in an amino acid sequence of SEQ ID NO: 1. The invention also provides for an isolated mutant human von Willebrand Factor A1 protein consisting of a 1472H>Q mutation in an amino acid sequence of SEQ ID NO: 1. The invention also provides for an isolated mutant human von Willebrand Factor A1 protein consisting of a 1473M>V mutation in an amino acid sequence of SEQ ID NO: 1. The invention also provides for an isolated mutant human von Willebrand Factor A1 protein consisting of a 1475Q>H mutation in an amino acid sequence of SEQ ID NO: 1. The invention also provides for an isolated mutant human von Willebrand Factor A1 protein consisting of a 1479G>S mutation in an amino acid sequence of SEQ ID NO: 1.

[0075] The invention also provides for an isolated mutant human von Willebrand Factor A1 protein having SEQ ID NO: 6, wherein the protein comprises a mutation selected from the group consisting of: 1263S>P, 1269D>N, 1274R>K, 1287R>M, 1302D>G, 1308R>H, 1313W>R, 1314V>I, 1326H>R, 1329I>L, 1330G>E, 1333D>A, 1344A>T, 1347V>I, 1350A>T, 1370S>G, 1379R>H, 1381A>T, 1385M>T, 1391Q>P, 1394S>A, 1397F>L, 1421N>S, 1439V>L, 1442S>G, 1449Q>R, 1466P>A, 1469L>Q, 1472H>Q, 1473M>V, 1475Q>H, or a 1479G>S.

[0076] The invention provides for a transgenic non-human animal expressing von Willebrand Factor A1 protein containing mutation(s) at one of more amino acid position selected from the group consisting of: 1263, 1269, 1274, 1287, 1302, 1308, 1313, 1314, 1326, 1329, 1330, 1333, 1344, 1347, 1350, 1370, 1379, 1381, 1385, 1391, 1394, 1397, 1421, 1439, 1442, 1449, 1466, 1469, 1472, 1473, 1475, and 1479, wherein the position corresponds to an amino acid position of human von Willebrand Factor A1 protein shown in SEQ ID NO: 6. In one embodiment, the animal is a murine, a porcine, a canine, a feline, a rabbit, or a primate. In another embodiment, the animal is a mouse, a rat, a dog, a sheep, a goat, a horse, a cow, a cat, a monkey, a primate, a pig, a llama, an alpaca, a chicken, etc. In other embodiments, the protein comprises a single mutation. In further embodiments, the protein comprises two or more mutations. In yet another embodiment, the protein comprises at least one mutation selected from the group consisting of: 1263>S, 1269>D, 1274>R, 1287>R, 1302>D, 1308>R, 1313R>W, 1314>V, 1326>H, 1329>I, 1330>G, 1333>D, 1344>A, 1347>V, 1350>A, 1370>S, 1379>R, 1381>A, 1385>M 1391>Q, 1394>S, 1397>F, 1421>N, 1439>V, 1442>S, 1449>Q, 1466>P, 1469>L, 1472>H, 1473>M, 1475>Q, 1479>G, and any combination thereof. In particular embodiments, the protein comprises a 1326R>H mutation. In other embodiments, the protein comprises a 1314I>V mutation. In yet other embodiments, the protein comprises a 1326R>H mutation, a 1314I>V mutation, or a combination of the two mutations listed previously. In some embodiments of the invention, the animal is a mouse. In further embodiments, the protein comprises SEQ ID NO: 5. In other embodiments, the VWF protein is at least 5%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 99%, or 100% identical to the A1 domain of human VWF protein as shown in SEQ ID NO: 1. In particular embodiments, the von Willebrand Factor A1 protein of the transgenic animal comprises the human A1 domain shown in SEQ ID NO: 1. In some embodiments, the von Willebrand Factor A1 protein is partially or completely replaced with a human von Willebrand Factor A1 protein comprising SEQ ID NO: 1. In other embodiments, the animal is a model for pre-clinical testing of compounds that expresses a mutant von Willebrand Factor (VWF) A1 protein containing one or more mutations, wherein the binding specificity of the mutant VWF-A1 protein changes from being specific for the animal platelets to being specific for human platelets. In further embodiments the mutant VWF-A1 protein in the animal binds to human platelets.

[0077] The invention is provides a method for identifying a compound that modulates binding of VWF-A1 protein to GPIb-alpha protein. The method comprises: providing an electronic library of test compounds; providing atomic coordinates listed in Table 8 for at least 10 amino acid residues for the A1 domain of the VWF protein, wherein the coordinates have a root mean square deviation therefrom, with respect to at least 50% of Cα atoms, of not greater than about 2.5 Å, in a computer readable format; converting the atomic coordinates into electrical signals readable by a computer processor to generate a three dimensional model of the VWF-A1 domain; performing a data processing method, wherein electronic test compounds from the library are superimposed upon the three dimensional model of the VWF-A1 domain; and determining which test compound fits into the binding pocket of the three dimensional model of the VWF-A1 protein, thereby identifying which compound would modulate the binding of VWF-A1 protein to GPIb-alpha protein. Alternatively, the method can comprise: providing an electronic library of test compounds; providing atomic coordinates listed in Table 8 in a computer readable format for at least 10, 15, 20, 25, 30, 35, or 40 amino acid residues for the A1 domain of the VWF protein, wherein the residues comprise two or more of the following residues: Pro1391, Arg1392, Arg1395, Val1398, Arg1399, Gln1402, Lys1406, Lys1423, Gln1424, Leu1427, Lys1430, or Glu1431; converting the atomic coordinates into electrical signals readable by a computer processor to generate a three dimensional model of the VWF-A1 domain; performing a data processing method, wherein electronic test compounds from the library are superimposed upon the three dimensional model of the VWF-A1 domain; and determining which test compound fits into the binding pocket of the three dimensional model of the VWF-A1 protein, thereby identifying which compound would modulate the binding of VWF-A1 protein to GPIb-alpha protein.

[0078] In one embodiment, determining comprises detecting an IC_{50} of less than about 7.5 μg/ml for a test compound. In another embodiment, the method can further comprise: obtaining or synthesizing a compound; contacting VWF-A1 protein with the compound under a condition suitable for GPIb-alpha-VWF-A1 binding; and determining whether the compound modulates GPIb-alpha-VWF-A1 protein binding using a diagnostic assay. In a further embodiment, contacting comprises perfusing platelets into a flow chamber at a shear flow rate of at least 100 s^{-1}, wherein mutant murine VWF-A1 protein is immobilized on a bottom surface of the chamber, while in another embodiment contacting comprises perfusing platelets into the transgenic non-human animal described above. In some embodiments, contacting occurs sequentially. In other embodiments, the perfusing of platelets occurs prior to administration of the compound. In one embodiment, the platelets are human platelets, while in other embodiments, the platelets are not murine platelets. In further embodiments, the determining comprises detecting an increase or decrease in the dissociation rate between VWF-A1 protein and GPIb-alpha protein by at least two-fold. In other embodiments, the determining comprises detecting an increase or decrease of platelet adhesion to a surface expressing VWF-A1 protein, while in some embodiments the determining comprises detecting an increase or decrease in a stabilization of an interaction between VWF-A1 protein and GPIb-alpha protein. In particular embodiments, the determining comprises detecting thrombosis formation. In some embodiments, the determining comprises identifying an occurrence of an abnormal thrombotic event in the subject. In further embodiments of the invention, an abnormal thrombotic event comprises abnormal bleeding, abnormal clotting, death, or a combination of the events listed. In some embodiments, the determining comprises dynamic force microscopy, a coagulation factor assay, a platelet adhesion assay, thrombus imaging, a bleeding time assay, aggregometry, review of real-time video of blood flow, a Doppler ultrasound vessel occlusion assay, or a combination thereof. In particular embodiments of the invention, perfusing platelets is followed by perfusion of a labeled agent. In some embodiments, the labeled agent comprises one or more of a nanoparticle, a fluorophore, a quantum dot, a microcrystal, a radiolabel, a dye, a gold biolabel, an antibody, or a small molecule ligand. In other embodiments, the agent targets a platelet receptor, a VWF protein, or a portion thereof. In another embodiment, the

animal is injected with nanoparticles, and/or peptides, and/or small molecules, which label the human platelets, at some time prior testing, wherein the nanoparticles, and/or peptides, and/or small molecules are capable of being imaged while in the animal. In another embodiment, the testing comprises tracking of human platelet deposition in the animal. In another embodiment, the compound or agent is an anti-thrombotic, such as an anti-platelet drug, e.g., PLAVIX, an ADP inhibitor, and/or a humanized antibody and/or small molecule that inhibits human alpha IIb and/or beta 3 integrin function, human alpha2 and/or beta 1 integrin function, human glycoprotein VI (GPVI) function, human thrombin receptors function, and/or intracellular signaling pathways (for example, phosphoinositide 3-kinases (PI3K)) vital to platelet function in hemostasis and thrombosis.

[0079] The invention provides for a method for testing a compound or agent, the method comprising: (a) providing a candidate agent or compound to be tested; (b) administering the agent or compound to an animal in an effective amount, wherein the animal expresses a mutant von Willebrand Factor A1 protein containing a mutation, combination of mutations that change the animal protein's binding specificity from being specific for animal platelets to being specific for human platelets, so that the mutant VWF-A1 protein in the animal binds to human platelets, and wherein the animal is perfused with human platelets; (c) testing the animal to determine whether the animal experiences any abnormal hemostatic and/or thrombotic events, thereby testing the compound or agent.

[0080] The invention provides for a nucleic acid encoding the mutant von Willebrand Factor A1 protein of the invention. The invention provides for a vector containing such a nucleic acid. The invention provides for an animal expressing such a nucleic acid to express the mutant VWF protein.

[0081] The invention also provides a method for treating von Willebrand Disease (VWD) in a subject in need thereof, the method comprising administering to the subject an effective amount of a compound that promotes platelet adhesion in the subject, wherein the compound increases the dissociation rate between VWF-A1 protein and GPIb-alpha protein by at least two-fold, thereby administration of the compound increases blood coagulation in the subject. In one embodiment, coagulation is measured by a coagulation factor assay, an ex-vivo flow chamber assay, or a combination thereof.

[0082] The invention provides a method for rapidly detecting an internal vascular injury site in a subject. The method comprises: administering to a subject a targeted molecular imaging agent, wherein the molecule circulates for an effective period of time in order to bind to the injury site within the subject; tracking a deposition of the labeled targeted molecular imaging agent in the subject; and identifying the site of a thrombus formation in the subject by imaging the targeted molecular imaging agent, thereby the deposition of the targeted molecular imaging agent at the internal vascular injury site is indicative of internal bleeding within a subject. In one embodiment, the targeted molecular imaging agent is administered by subcutaneous, intra-muscular, intra-peritoneal, or intravenous injection; infusion; by oral, nasal, or topical delivery; or a combination of the routes listed. In another embodiment, the targeted molecular imaging agent comprises a nanoparticle, a fluorophore, a quantum dot, a microcrystal, a radiolabel, a dye, a gold biolabel, an antibody, a peptide, a small molecule ligand, or a combination of the agents listed. In a further embodiment, the nanoparticle comprises a perfluorocarbon, while in particular embodiments, the nanoparticle is coupled to an antibody, a small molecule, a peptide, or a receptor trap. In some embodiments, the targeted molecular imaging agent specifically binds to a platelet receptor, or a VWF protein, or a portion thereof. In other embodiments, the targeted molecular imaging agent has a $T_{1/2}$ of at least 30 minutes. In further embodiments, imaging comprises a PET scan, MRI, IR scan, ultrasound, nuclear imaging, or a combination of the methods listed. In a particular embodiment, the subject is further administered a pro-thrombotic compound. In a further embodiment, the compound increases the dissociation rate between VWF-A1 protein and GPIb-alpha protein by at least two-fold.

[0083] Considerable emphasis has been placed on elucidating the role of structural changes in the A1 domain of VWF in order to gain insight into mechanism(s) that may regulate its binding to platelet GPIb alpha. This invention concerns the contribution of the biophysical properties of this interaction in governing platelet adhesion under hydrodynamic conditions. It has been demonstrated that flow-dependent adhesion and rapid and force-driven kinetic properties define the GPIb alpha-VWF-A1 bond. The invention provides classification of subtypes of von Willebrand disease (vWD), such as platelet-type, type 2B or type 2M in terms of similarities in the alterations in the biophysical properties of bonds to better understand the clinical phenotypes associated with these bleeding disorders. The invention is directed to understanding the biomechanical and molecular mechanisms by which the VWF-A1 domain mediates adhesive interactions with GPIb alpha both in vitro and in vivo. This invention provides for mice with mutant A1 domains to demonstrate the importance of the intrinsic kinetic and mechanical properties of this receptor-ligand pair in preventing inappropriate platelet aggregation in circulating blood, to facilitate the study of human platelet biology, as well as to generate a humanized animal model of hemostasis and thrombosis. The animal model is also useful for the generation and testing of novel anti-thrombotic therapies designed to inhibit platelet-VWF interactions as well as those designed to correct the bleeding phenotype associated with a reduction in adhesion between this receptor-ligand pair.

[0084] The invention is directed to understanding the effect that alterations in platelet size and shape have on the force-driven kinetics of the GPIb alpha-VWF-A1 tether bond. Platelets can bind to and translocate on surface-immobilized VWF under shear forces that preclude selectin-dependent adhesion of leukocytes to the vessel wall, due to their small discoid shape and not as a consequence of the unique kinetic properties of the GPIb alpha-VWF-A1 tether bond. The contribution of particle geometry in supporting the interactions between this receptor-ligand pair, we is performed by evaluating the interaction between vWF-A1 coated microspheres, ranging from 4 to 12 μm in diameter (platelet to leukocyte size), with surface-immobilized platelets under physiologic flow conditions. The utility of using polystyrene microspheres with a uniform shape and size to permit determination of the relationship between wall shear stress and the force acting on the GPIb alpha-vWF-A1 tether bond has been demonstrated. Moreover, it has been shown that the "sidedness" of the receptor or ligand does not alter the kinetic properties of this bond. β-tubulin deficient mice have a defect in the cytoskeleton of platelets that changes the shape of these cells from discoid to spherical. Preliminary data demonstrates that the "spherical" platelets have a >60% reduction in attachment at

high shear rates as compare to wild-type (WT) platelets. No differences in the kinetics of the GPIb alpha-vWF-A1 tether bond for WT and mutant platelets have been found using our microsphere assay system. Thus, platelet shape and not alterations in the biophysical properties of the GPIb alpha-vWF-A1 tether bond are responsible for the defect in adhesion. Data generated from these experiments will be use to develop a computational algorithm designed to stimulate the adhesion of platelets to surface-immobilized vWF under various hydrodynamic conditions. Thus, the invention provides an in vivo method to test for defects in hemostasis and thrombus formation that result from abnormalities in platelet shape and size. It also provides for a method to test the ability of synthetic platelet substitutes, which may be of varying shapes and sizes, to support hemostasis.

[0085] The invention provides a method for determining whether platelet function or morphology in a subject is abnormal. The method comprises: affixing a molecule comprising a murine VWF-A1 domain to a surface of a flow chamber, wherein the domain comprises at least one mutation at a position selected from the group consisting of 1263>S, 1269>D, 1274>R, 1287>R, 1302>D, 1308>R, 1313R>W, 1314>V, 1326>H, 1329>I, 1330>G, 1333>D, 1344>A, 1347>V, 1350>A, 1370>S, 1379>R, 1381>A, 1385>M 1391>Q, 1394>S, 1397>F, 1421>N, 1439>V, 1442>S, 1449>Q, 1466>P, 1469>L, 1472>H, 1473>M, 1475>Q, 1479>G, and any combination thereof, where the position corresponds to an amino acid position of human von Willebrand Factor A1 protein shown in SEQ ID NO: 6; perfusing through the flow chamber a volume of blood or plasma from a subject at a shear flow rate of at least about 100 s^{-1}; perfusing a targeted molecular imaging agent into the flow chamber; and comparing the flow rate of the blood or plasma from the subject as compared to a normal flow rate, so as to determine whether the subject's platelet function or morphology is abnormal.

[0086] In one embodiment, affixing comprises (i) affixing an antibody which specifically binds VWF-A1 domain, and (ii) perfusing murine mutant VWF-A1 protein in the flow chamber at a shear flow rate of at least 100 s^{-1}. In another embodiment, the targeted molecular imaging agent comprises a nanoparticle, a fluorophore, a quantum dot, a microcrystal, a radiolabel, a dye, a gold biolabel, an antibody, a peptide, a small molecule ligand, or a combination of the agents listed. In a further embodiment, the targeted molecular imaging agent binds to a platelet receptor, a platelet ligand, or any region of a VWF protein or a portion thereof. In a particular embodiment, the targeted molecular imaging agent comprises horseradish peroxidase (HRP) coupled to an antibody directed at VWF-A1. In other embodiments, the comparing comprises a platelet adhesion assay, fluorescence imaging, a chromogenic indicator assay, a microscopy morphology analysis, or any combination of the listed modes. In some embodiments, platelets bound to VWF-A1 are less than about 500 cells/mm^2. In particular embodiments, the platelets are substantially spherical. In further embodiments, the subject is a human, a canine, a feline, a murine, a porcine, an equine, or a bovine. In other embodiments, the VWF molecule is an antibody, a peptide, or a Fab fragment directed to a VWF polypeptide or a portion thereof.

[0087] The invention also provides for a method for producing von Willebrand Factor A1 protein that specifically binds human platelets, the method comprising: (a) providing an animal expressing a plasma VWF containing a mutant von Willebrand Factor A1 protein, wherein the mutation causes the animal's von Willebrand Factor A1 protein to bind preferentially to for human platelets; and (b) harvesting the mutant animal von Willebrand Factor A1 so as to produce von Willebrand Factor A1 protein that specifically binds human platelets. In one embodiment, the mutant animal von Willebrand Factor A1 protein comprises at least one mutation comprising 1263P>S, 1269N>D, 1274K>R, 1287M>R, 1302G>D, 1308H>R, 1313R>W, 1314I>V, 1326R>H, 1329L>I, 1330E>G, 1333A>D, 1344T>A, 1347I>V, 1350T>A, 1370G>S, 1379H>R, 1381T>A, 1385T>M 1391P>Q, 1394A>S, 1397L>F, 1421S>N, 1439L>V, 1442G>S, 1449R>Q, 1466A>P, 1469Q>L, 1472Q>H, 1473V>M, 1475H>Q, 1479S>G, or any combination thereof.

[0088] The invention provides a method for testing efficacy and toxicity of a gene therapy vector, wherein the method comprises: (a) introducing a gene therapy vector into the animal of claim **4**, allowing sufficient time for expression of the vector; (b) perfusing platelets from a subject into the animal under a condition suitable for GPIb-alpha-VWF-A1 protein binding; and (c) determining whether or not a thrombotic event occurs in the animal. In one embodiment, the vector comprises a nucleic acid encoding a platelet receptor polypeptide, a platelet ligand polypeptide, or a VWF polypeptide, or a portion thereof. In another embodiment, the subject is a human, a dog, a cat, a horse, a pig, or a primate. In a particular embodiment, the platelets are not murine platelets. In a further embodiment, the thrombotic event comprises blood clotting, abnormal bleeding, abnormal clotting, death, or a combination thereof. In some embodiments, the determining comprises dynamic force microscopy, a coagulation factor assay, a platelet adhesion assay, thrombus imaging, a bleeding time assay, aggregometry, review of real-time video of blood flow, a Doppler ultrasound vessel occlusion assay, or a combination thereof. In other embodiments, perfusing platelets is followed by perfusion of a labeled agent. In further embodiments, the labeled agent comprises one or more of a nanoparticle, a fluorophore, a quantum dot, a microcrystal, a radiolabel, a dye, a gold biolabel, an antibody, or a small molecule ligand. In particular embodiments, the agent targets a platelet receptor, a VWF protein, or a portion thereof.

[0089] The invention also provides a method for calibrating an aggregometry device or a device for measuring clot formation or retraction, wherein the method comprises: (a) providing hematologic data obtained from a subject, wherein blood or platelets from the subject is assessed by the device; (b) determining whether or not a thrombotic event occurs in the animal described above, wherein the animal is perfused with a sample of blood or platelets from the subject; and (c) correlating data obtained from (b) with the data obtained in (a) so as to calibrate the device, wherein a certain data obtained from the device is indicative of the corresponding thrombotic outcome determined in the animal described above. In one embodiment, the thrombotic event comprises blood clotting, abnormal bleeding, abnormal clotting, death, or a combination thereof.

[0090] The invention provides for in vivo determination of intrinsic and mechanical properties of the GPIb alpha-vWF-A1 tether bond and to determine if they are indeed critical for regulating the adhesion between platelets and vWF and how they may be manipulated to impair or enhance hemostasis and/or thrombosis. The invention provides determination of whether animals that posses gain-of-function mutations in VWF-A1, for example, those associated with type 2B vWD,

have a defect in platelet deposition at sites of vascular injury and/or a loss of high molecular weight multimers of vWF. The invention provides determination of whether animals that possess loss-of-function mutations in VWF-A1, for example, those associated type 2M vWD, have a defect in platelet deposition at sites of vascular injury. Based on the results obtained herein, mice are genetically engineered with 1) mutant A1 domains that increase or decrease the on- and/or off-rate of this receptor-ligand pair by varying degrees, 2) A1 domains containing both types of mutations to confirm whether specific regions within this domain are essential for the stabilization of GPIb alpha binding, and 3) mutations within the A1 domain that favor binding to human but not murine GPIb alpha to enable the study of human platelet behavior in an animal model of hemostasis or thrombosis.

[0091] The invention provides methods for determining the impact of altering the intrinsic bond kinetics and/or its mechanical properties of the GPIb alpha-VWF-A1 interaction on hemostasis and thrombosis, which comprises: measuring platelet counts, plasma levels of vWF, and bleeding times; performing multimer gel analysis of mutant vWF; measuring the affinity of mutant vWF for platelets using a fluid phase binding assay; evaluating in vitro platelet tethering, rolling, and thrombus formation on surface-immobilized murine plasma vWF containing mutant A1 domains under physiological flow conditions; determining the ability of thrombi to form at sites of vascular injury in vivo in mutant VWF mice using epifluorescent intravital microscopy in; measuring platelet tethering frequency and rolling velocities in vivo.

[0092] In certain embodiments, the subject is a human. In other embodiments, the subject is a non-human animal such as a canine, equine, feline, porcine, murine, bovine, foul, sheep, or any other animal in need of treatment. In certain embodiments, the pharmaceutical composition further comprises another active agent. The additional active agent can be, but is not limited to, an analgesic, an antioxidant, diuretic, or a combination thereof. In certain embodiments, the composition is in a capsule form, a granule form, a powder form, a solution form, a suspension form, a tablet form, or any other form suitable for use by the method of the present invention. In certain embodiments, the composition is administered via oral, sublingual, buccal, parenteral, intravenous, transdermal, inhalation, intranasal, vaginal, intramuscular, rectal administration or any other route of administration that is suitable for delivery of the compound.

DETAILED DESCRIPTION OF THE INVENTION

[0093] The invention provides methods for identifying and evaluating potential anti-thrombotic reagents and compounds. The invention provides methods for testing for undesirable thrombotic or bleeding side effects of reagents in the setting of preclinical testing. The invention provides an in vivo model to test the efficacy of potential anti-thrombotic drugs directed against receptors, ligands, and/or intracellular signaling pathways on or in human platelets prior to FDA approval. To date, in vitro models of thrombosis do not accurately recapitulate the hemodynamic conditions, cell-cell interactions, or cell-protein interactions that occur at sites of vascular injury in a living animal. Thus, the system provided by this invention provides a method to test drugs directed at inhibiting or altering human platelet function other than directly testing them in humans. The invention provides a great advantage of being able to test directly compounds that target human platelets in an in vivo system. The invention provides a method to test contrast agents for imaging of human platelets at sites of thrombosis. For instance, one could test the ability of nanoparticle contrast agents targeted to human platelets to identify areas of thrombosis or occult bleeding. The invention provides a method to test compounds that correct the bleeding phenotype associated with a reduction in interactions between GPIb alpha and VWF-A1. The invention provides a method to test gene therapies directed at correcting genetic mutations associated with von Willebrand disease. The invention provides a method to correlate results obtained with an in vitro assay designed to measure the effects of antithrombotics or markers of platelet activation in patients.

[0094] Terms

[0095] A "pharmaceutical composition" refers to a mixture of one or more of the compounds, or pharmaceutically acceptable salts, hydrates, polymorphs, or pro-drugs thereof, with other chemical components, such as physiologically acceptable carriers and excipients. The purpose of a pharmaceutical composition is to facilitate administration of a compound to an organism.

[0096] As used herein von Willebrand factor is abbreviated "VWF" and, alternatively, "vWF".

[0097] A "pro-drug" refers to an agent which is converted into the parent drug in vivo. Pro-drugs are often useful because, in some situations, they are easier to administer than the parent drug. They are bioavailable, for instance, by oral administration whereas the parent drug is not. The pro-drug also has improved solubility in pharmaceutical compositions over the parent drug. For example, the compound carries protective groups which are split off by hydrolysis in body fluids, e.g., in the bloodstream, thus releasing active compound or is oxidized or reduced in body fluids to release the compound.

[0098] A compound of the present invention also can be formulated as a pharmaceutically acceptable salt, e.g., acid addition salt, and complexes thereof. The preparation of such salts can facilitate the pharmacological use by altering the physical characteristics of the agent without preventing its physiological effect. Examples of useful alterations in physical properties include, but are not limited to, lowering the melting point to facilitate transmucosal administration and increasing the solubility to facilitate administering higher concentrations of the drug.

[0099] The term "pharmaceutically acceptable salt" means a salt, which is suitable for or compatible with the treatment of a patient or a subject such as a human patient or an animal.

[0100] The term "pharmaceutically acceptable acid addition salt" as used herein means any non-toxic organic or inorganic salt of any base compounds of the invention or any of their intermediates. Illustrative inorganic acids which form suitable acid addition salts include hydrochloric, hydrobromic, sulfuric and phosphoric acids, as well as metal salts such as sodium monohydrogen orthophosphate and potassium hydrogen sulfate. Illustrative organic acids that form suitable acid addition salts include mono-, di-, and tricarboxylic acids such as glycolic, lactic, pyruvic, malonic, succinic, glutaric, fumaric, malic, tartaric, citric, ascorbic, maleic, benzoic, phenylacetic, cinnamic and salicylic acids, as well as sulfonic acids such as p-toluene sulfonic and methanesulfonic acids. Either the mono or di-acid salts can be formed and such salts exist in either a hydrated, solvated or substantially anhydrous form. In general, the acid addition salts of compounds of the

invention are more soluble in water and various hydrophilic organic solvents, and generally demonstrate higher melting points in comparison to their free base forms. The selection of the appropriate salt will be known to one skilled in the art. Other non-pharmaceutically acceptable salts, e.g., oxalates, are used, for example, in the isolation of compounds of the invention for laboratory use or for subsequent conversion to a pharmaceutically acceptable acid addition salt.

[0101] As used herein and as well understood in the art, "treatment" is an approach for obtaining beneficial or desired results, including clinical results. Beneficial or desired clinical results can include, but are not limited to, alleviation or amelioration of one or more symptoms or conditions, diminishment of extent of disease, stabilized (i.e., not worsening) state of disease, preventing spread of disease, delay or slowing of disease progression, amelioration or palliation of the disease state and remission (whether partial or total), whether detectable or undetectable. "Treatment" can also mean prolonging survival as compared to expected survival if not receiving treatment.

[0102] Arterial thrombosis is a pathological consequence of disease states such as atherosclerosis and remains a major cause of morbidity and mortality in the Western world with healthcare cost ranging in the billions of dollars in the USA alone (Circulation 2006; 113:e85). Central to this process is the inappropriate deposition and activation of platelets in diseased vessels that can ultimately occlude the lumen, thus impeding blood flow to vital organ such as the heart and brain. Another key player is VWF, a large plasma glycoprotein of complex multimeric structure, which under normal physiological conditions prevents excessive bleeding by promoting platelet deposition at sites of vascular injury, thus "sealing off" leaky blood vessels. In order for this event to occur, VWF must form a "bridge" between receptors expressed on circulating platelets and exposed components of the injured vessel wall. This is the function of the A1 and A3 domains of this plasma protein, respectively. Each is folded into a disulfide-bonded loop structure that is critical for optimal biological activity (FIG. **1**A).

[0103] It is the A1 domain that contains residues that compose the binding site for its receptor on platelets known as GPIb alpha, an adhesive event essential for the ability of these cells to rapidly attach to the injured vessel wall. The critical nature of this interaction is exemplified by the bleeding disorder, termed type 2M von Willebrand Disease (VWD), which results from the incorporation of loss-of-function point mutations within this domain that reduce the interaction between VWF-A1 and GPIb alpha (Sadler J E et al.(2006) *J. Thromb. Haemost.* 4: 2103-14). The A3 domain, on the other hand, is believed to be important in anchoring plasma VWF to sites where extracellular matrix components (i.e. collagen) are exposed as a result of disruption of the overlying vasculature endothelium (Wu D. et al. *Blood* 2002). Once in contact with exposed elements of the damaged vessel wall, platelets become "activated" through various signaling pathways (i.e. GPVI) enabling other adhesion molecules, such as $\alpha 2\beta 1$ (collagen receptor) and $\alpha IIb\beta 3$ (fibrinogen and VWF receptor) integrins, to firmly anchor these cells at the site of injury and to each other (FIG. **1**B). In addition, ADP released from adherent platelets serves to amplify the activation of integrin receptors as well as other platelets leading to thrombus growth. Considerable emphasis has been placed on understanding the mechanism(s) that govern the interaction between GPIb alpha and the A1 domain of VWF and how it can be perturbed by point mutations associated with von Willebrand Disease, information relevant to the development of anti-thrombotic therapies.

[0104] von Willebrand Factor (VWF), the A1 Domain, and Related Diseases

[0105] During the past two decades, there has been considerable progress in understanding how VWF mediates platelet adhesion. Both the VWF cDNA and gene have been cloned and the primary structure of the VWF subunit (FIG. **1**A) has been determined (13-16). It has been reported that ~59% of the mature VWF consists of repeated segments which are 29% to 43% homologous (17). These regions consist of domains that are triplicated (domains "A" and "B"), duplicated (domain "C") or quadruplicated (domain "D"). The triplicated A repeats encode for the central region of each VWF subunit. The A1 and A3 domains contain the sequences that mediate VWF's interaction with receptors on platelets or components of subendothelial extracellular matrix, respectively. Each is folded into a disulfide-bonded loop structure that is critical for optimal biological activity. The sequences of the amino terminal halves of each loop and the location of the cysteines forming the loop structure of each domain are highly conserved. It is the VWF-A1 domain (1260-1480) that contains sequences that provide binding sites for the platelet glycoprotein receptor Ib alpha, an interaction critical for the ability of these cells to rapidly attach and translocate at sites vascular injury (18,19). The role of the A3 domain, however, is believed to be in anchoring plasma VWF at sites where extracellular matrix components (i.e. collagen) are exposed as a result of disruption of the endothelium (20-25).

[0106] With regard to mediating adhesive interactions with platelets, it has become increasingly evident that the VWF-A1 domain plays a crucial role in this process based on molecular genetic studies of individuals with type 2M or 2B VWD (26-31). In the majority of cases, patients with these designated genotypes have single point mutations contained within the disulfide loop (between Cys 1272 and Cys 1458) of this domain. With regard to type 2M VWD, afflicted individuals have significant impairments in hemostasis that appears to result from a lack of or reduced adhesive interactions between GPIb alpha and VWF at sites of vascular injury, and not from an alteration in VWF multimer structure. Structural and functional evidence has been provided in that type 2M mutations, such as 1324G>S, are localized within a region of the A1 domain (FIG. **5**B), which is critical for supporting GPIb alpha-mediated platelet adhesion at physiological flow rates. Confirmation that this residue, as well as others predicted by our analysis of the crystal structure of the A1 domain, does contribute to GPIb alpha binding is suggested by studies evaluating the structure of the complex formed between this receptor-ligand pair (32, 33). Thus, it is possible to make accurate predictions about protein function from the three-dimensional protein structure and to confirm these postulates by site-specific mutagenesis and analysis under physiologically relevant flow conditions. The localization of some of the residues within the A1 domain that when mutated disrupt GPIb alpha binding is shown below (FIG. **2**). The invention provides methods for evaluating the effect that loss-of-function mutations have on hemostasis and thrombus formation.

[0107] In contrast type 2M VWD, mutations associated with type 2B VWD are known to enhance the interaction between VWF-A1 and GPIb alpha, that is, they mitigate the requirement for exogenous modulators such as ristocetin or botrocetin to induce platelet agglutination (30). Moreover,

these altered residues are localized in a region remote from the major GPIb alpha binding site that has been identified by mutagenesis (26; FIG. **3**A—yellow). Clinically, this disease state is characterized by a loss of circulating high molecular weight VWF multimers (HMWM, FIG. **3**B) together with a mild to moderate thrombocytopenia, which ultimately results in bleeding but not thrombosis (30, 31). It is the clearance of the HMWM of VWF from plasma that is believed to be responsible for the increased bleeding tendencies in patients with this disorder as they contribute to the majority of the hemostatic function associated with this plasma glycoprotein (34). The invention provides methods for evaluating the effect that gain-of-function mutations have on hemostasis, thrombus formation, and plasma levels of VWF.

[0108] Surface-immobilization of VWF and subsequent exposure to physiologically relevant shear forces appears to be a prerequisite for its ability to support interactions with platelets as this multimeric protein does not bind appreciably to these cells in the circulation. These hydrodynamic conditions are believed to promote structural changes within the A1 domain that in turn increases its affinity for GPIb alpha (35-37). Evidence suggested to support the existence of such an alteration in structure includes the ability of non-physiologic modulators such as the antibiotic ristocetin or the snake venom protein botrocetin to promote platelet agglutination in solution-based assays (38, 39). Moreover, this "on" and "off" conformation is exemplified by type 2B VWD. For instance, it was initially hypothesized that incorporation of type 2B mutations into the A1 domain shifted the equilibrium between two distinct tertiary conformations, analogous to those seen in crystal structures of the integrin I domain in ligand-free and collagen-bound states (40). The location of the type 2B mutants at sites distinct from the GPIb alpha binding site suggest that they disrupt a region responsible for regulation of binding affinity, thus affecting ligand binding allosterically. The crystal structure of a type 2B mutant A1 domain, 1309I>V, was determined and compared to its wild-type counterpart (41). A change was discovered in the structure of a loop, thought to be involved in GPIb alpha binding, lying on the surface distal to the mutation site. A similar finding has been observed for the VWF-A1 crystal structure containing the identical mutation (FIG. **4**).

[0109] This altered conformation represents a high affinity binding state of the A1 domain. However, the pathway of allosteric change proposed previously, involving the burial of a water molecule, cannot be a general feature of type 2B mutants, and the structural rearrangements appear too subtle to explain the altered kinetics. Interestingly, complex formation between botrocetin and VWF-A1 in which the type 2B mutation 1309I>V has been incorporated, and has demonstrated that most of these structural differences are reversed including: (1) loss of the buried water molecule at the mutation site; (2) the peptide plan between Asp 1323 and Gly 1324 flips back to a conformation similar to that in the WT structure; and (3) the side chain of His 1326 remains in the "mutant" position, although there is some evidence from electron density of an alternative conformation similar to WT. However, this reversion in structure does not correlate with a loss in the function-enhancing activity associated with the type 2B mutation. In fact, the addition of botrocetin further augments the interaction between the mutant A1 domain and platelets in flow (42). Thus, an alternative mechanism must account for function-enhancing nature of type 2B mutations. Moreover, these subtle alterations in structure did not compare to the large conformational changes in homologous integrin I-type domains that occur on ligand binding.

[0110] Recent findings provide support that type 2B mutations may stabilize the binding of a region of GPIb alpha known as the β-hairpin to an area near the location of these altered residues, distinct from that identified by site-directed mutagenesis. Type 2B mutations have been suggested to destabilize a network of interactions observed between the bottom face of the A1 domain and its terminal peptides in the wild type A1 structure, thereby making the binding site accessible (FIG. **6**).

[0111] Progress has been made in understanding the structure of this receptor-ligand pair and potential alterations in conformation that may regulate this interaction. This model is non-limiting. However, the model permits determination of the kinetic and biomechanical basis for 1) the regulation of VWF-A1 domain activity in response to hydrodynamic forces, 2) the alterations in bond kinetics that result from incorporation of type 2B mutations into the VWF-A1 domain, and 3) the susceptibility of the kinetics of the GPIb alpha-VWF-A1 bond to an applied force.

[0112] Transgenic Animals

[0113] The invention provides for transgenic non-human animals that comprise a genome contains a nucleotide sequence (such as a gene) encoding a modified form of the A1 domain of VWF. The modification can be an amino acid residue substitution at a position involved with binding to GPIb alpha or in close proximity to this region such as, but not limited to, positions 1263, 1269, 1274, 1287, 1302, 1308, 1313, 1314, 1326, 1329, 1330, 1333, 1344, 1347, 1350, 1370, 1379, 1381, 1385 1391, 1394, 1397, 1421, 1439, 1442, 1449, 1466, 1469, 1472, 1473, 1475, 1479. It can also comprise a partial or full replacement of the animal A1 domain of VWF with the A1 domain of human VWF. It can also comprise a partial or full replacement of the entire VWF gene in an animal with the human VWF gene. Such non-human animals include vertebrates such as ovines, bovines, rodents, non-human primates, porcines, caprines, equines, ruminants, lagomorphs, canines, felines, aves, and the like. In one embodiment, non-human animals are selected from the order Rodentia that includes murines (such as rats and mice). In another embodiment, mice are particularly useful.

[0114] The transgenic non-human animals of the current invention are produced by experimental manipulation of the genome of the germline of the non-human animal (such as those animals described above). These genetically engineered non-human animals may be produced by several methods well known in the art which include the introduction of a "transgene" that comprises a nucleic acid (for example, DNA such as the A1 domain of VWF) integrated into a chromosome of the somatic and/or germ line cells of a non-human animal via methods known to one skilled in the art or into an embryonal target cell. A transgenic animal is an animal whose genome has been altered by the introduction of a transgene.

[0115] The term "transgene" as used herein refers to a foreign gene that is placed into an organism by introducing the foreign gene into newly fertilized eggs, embryonic stem (ES) cells, or early embryos. The term "foreign gene" refers to any nucleic acid (for example, a gene sequence) that is introduced into the genome of an animal by experimental manipulations. These nucleic acids may include gene sequences found in that animal so long as the introduced gene contains some modification (for example, the presence of a

selectable marker gene, a point mutation—such as the base pair substitution mutant that contributes to the amino acid change at amino acid residue 1326 of the A1 domain of VWF—the presence of a loxP site, and the like) relative to the naturally-occurring gene.

[0116] The term "loxP site" refers to a short (34 bp) DNA sequence that is recognized by the Cre recombinase of the *E. coli* bacteriophage P1. In the presence of Cre recombinase, placement of two loxP sites in the same orientation on either side of a DNA segment can result in efficient excision of the intervening DNA segment, leaving behind only a single copy of the loxP site (Sauer and Henderson (1988) *Proc. Natl. Acad. Sci. USA* 85:5166).

[0117] In one embodiment, the invention provides for a targeting construct or vector that comprises a selectable marker gene flanked on either side by a modified A1 domain of VWF. The modification of the A1 domain can comprise an amino acid residue substitution at a position involved with binding to GPIb alpha (such as, but not limited to, positions 1263, 1269, 1274, 1287, 1302, 1308, 1313, 1314, 1326, 1329, 1330, 1333, 1344, 1347, 1350, 1370, 1379, 1381, 1385 1391, 1394, 1397, 1421, 1439, 1442, 1449, 1466, 1469, 1472, 1473, 1475, 1479) or can be a partial or full replacement of an animal (for example, a mouse) A1 domain of VWF with the A1 domain of human VWF. The targeting vector contains the modified A1 domain of VWF gene sequence sufficient to permit the homologous recombination of the targeting vector into at least one allele of the A1 domain of the VWF gene resident in the chromosomes of the target or recipient cell (for example, ES cells). The targeting vector will usually harbor 10 to 15 kb of DNA homologous to the A1 domain of the VWF gene, wherein this 10 to 15 kb of DNA will be divided more or less equally on each side of the selectable marker gene. The targeting vector can contain more than one selectable maker gene and when multiple selectable marker genes are utilized, the targeting vector usually contains a negative selectable marker (for example, the Herpes simplex virus tk (HSV-tk) gene) and a positive selectable marker (such as G418 or the neo gene). The positive selectable marker permits the selection of recipient cells containing an integrated copy of the targeting vector and but does not enable one skilled in the art to determine whether this integration occurred at the target site or at a random site. The presence of the negative selectable marker permits the identification of recipient cells containing the targeting vector at the targeted site (for example, which has integrated by virtue of homologous recombination into the target site). Cells growing in medium that selects against the expression of the negative selectable marker represents that the cells do not contain a copy of the negative selectable marker.

[0118] Targeting vectors can also be of the replacement-type wherein the integration of a replacement-type vector results in the insertion of a selectable marker into the target gene. Replacement-type targeting vectors may be employed to disrupt a gene (such as the VWF gene or the A1 domain of the VWF gene). This can result in the generation of a null allele; for example, an allele not capable of expressing a functional protein wherein the null alleles may be generated by deleting a portion of the coding region, deleting the entire gene, introducing an insertion and/or a frameshift mutation, and the like.

[0119] A selectable marker can include a gene which encodes an enzymatic activity that confers resistance to an antibiotic or drug upon the cell in which the selectable marker is expressed. Selectable markers may be positive. A positive selectable marker is usually a dominant selectable marker wherein the genes encode an enzymatic activity that can be detected in a mammalian cell or a cell line (including ES cells). Some non-limiting examples of dominant selectable markers include the bacterial xanthine-guanine phosphoribosyl transferase gene (also referred to as the gpt gene) which confers the ability to grow in the presence of mycophenolic acid, the bacterial hygromycin G phosphotransferase (hyg) gene which confers resistance to the antibiotic hygromycin, and the bacterial aminoglycoside 3' phosphotransferase gene (also referred to as the neo gene) which confers resistance to the drug G418 in mammalian cells. Selectable markers may also be negative. Negative selectable markers encode an enzymatic activity whose expression is toxic to the cell when grown in an appropriate selective medium. One non-limiting example of a negative selectable marker is the HSV-tk gene wherein HSV-tk expression in cells grown in the presence of gancyclovir or acyclovir is catatonic. Growth of cells in selective medium containing acyclovir or gancyclovir therefore selects against cells capable of expressing a functional HSV TK enzyme.

[0120] The ES cells suitable of the present invention utilized to generate transgenic animals can harbor introduced expression vectors (constructs), such as plasmids and the like. The expression vector constructs can be introduced via transfection, lipofection, transformation, injection, electroporation, or infection. The expression vectors can contain coding sequences, or portions thereof, encoding proteins for expression. Such expression vectors can include the required components for the transcription and translation of the inserted coding sequence. Expression vectors containing sequences encoding the produced proteins and polypeptides, as well as the appropriate transcriptional and translational control elements, can be generated using methods well known to and practiced by those skilled in the art. These methods include in vitro recombinant DNA techniques, synthetic techniques, and in vivo genetic recombination which are described in J. Sambrook et al., 1989, *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Press, Plainview, N.Y. and in F. M. Ausubel et al., 1989, *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, N.Y. In one embodiment, loxP expressing targeting vectors are used for transfection methods (such as pDNR-1r vector, pACD4K-C vector, and the like). In other embodiments, Cre-recombinase-expressing plasmids are also utilized (for example, crAVE cre recombinase vectors).

[0121] Introducing targeting vectors into embryonic stem (ES) cells can generate the mutant VWF-A1 transgenic animals of the present invention. ES cells are obtained by culturing pre-implantation embryos in vitro under appropriate conditions (Evans, et al. (1981) *Nature* 292:154-156; Bradley, et al. (1984) *Nature* 309:255-258; Gossler, et al. (1986) *Proc. Acad. Sci. USA* 83:9065-9069; and Robertson, et al. (1986) *Nature* 322:445-448). Using a variety of methods known to those skilled in the art, transgenes can be efficiently introduced into the ES cells via DNA transfection methods, which include (but are not limited to), protoplast or spheroplast fusion, electroporation, retrovirus-mediated transduction, calcium phosphate co-precipitation, lipofection, microinjection, and DEAE-dextran-mediated transfection. Following the introduction into the blastocoel of a blastocyst-stage embryo, transfected ES cells can thereafter colonize an embryo and contribute to the germ line of the resulting chi-

meric animal (see Jaenisch, (1988) *Science* 240:1468-1474). Assuming that the transgene provides a means for selection, the transfected ES cells may be subjected to various selection protocols to enrich for ES cells that have integrated the transgene prior to the introduction of transfected ES cells into the blastocoel. Alternatively, the polymerase chain reaction (PCR) may be used to screen for ES cells that have integrated the transgene and precludes the need for growth of the transfected ES cells under appropriate selective conditions prior to transfer into the blastocoel.

[0122] Alternative methods for the generation of transgenic animals (such as transgenic mice) containing an altered A1 domain of the VWF gene are established in the art. For example, embryonic cells at various stages of development can be used to introduce transgenes for the production of transgenic animals and different methods are used that depend on the stage of embryonic cell development. For microinjection methods, the zygote is best suited. In the mouse, the male pronucleus reaches the size of approximately 20 microns in diameter, which allows for reproducible injection of 1-2 picoliters (pl) of suspended DNA solution. A major advantage in using zygotes as a gene transfer target is that in most cases the injected DNA will be incorporated into the host genome before the first cleavage (Brinster, et al. (1985) *Proc. Natl. Acad. Sci. USA* 82:4438-4442). Thus, all cells of the transgenic non-human animal (such as a mouse) will carry the incorporated transgene (mutant A1 domain of the VWF gene), which can result in the efficient transmission of the transgene to the offspring of the founder since 50% of the germ cells will harbor the transgene (see U.S. Pat. No. 4,873,191).

[0123] Another method known in the art that can be used to introduce transgenes into a non-human animal is retroviral infection. The developing non-human embryo can be cultured in vitro to the blastocyst stage wherein during this time, the blastomeres can be targets for retroviral infection (Janenich (1976) *Proc. Natl. Acad. Sci. USA* 73:1260-1264). Enzymatic treatment to remove the zona pellucida can increase infection efficiency of the blastomeres (Hogan et al. (1986) in *Manipulating the Mouse Embryo*, Cold Spring Harbor Laboratory Press, Plainview, N.Y.). The viral vector system used by one skilled in the art in order to introduce the transgene is usually a replication-defective retrovirus that harbors the transgene (Jahner, D. et al. (1985) *Proc. Natl. Acad. Sci. USA* 82:6927-6931; Van der Putten, et al. (1985) *Proc. Natl. Acad. Sci. USA* 82:6148-6152). Transfection can be easily and efficiently obtained via culturing blastomeres on a monolayer of virus-producing cells (Van der Putten, supra; Stewart, et al. (1987) *EMBO J.* 6:383-388). Infection can also be performed at a later stage whereby virus or virus-producing cells are injected into the blastocoele (Jahner, D. et al. (1982) *Nature* 298:623-628). Most of the founder non-human animals will be mosaic for the transgene since incorporation occurs only in a subset of cells that form the transgenic animal and the founder may additionally contain various retroviral insertions of the transgene at different positions in the genome that generally will segregate in the offspring. Additional methods of using retroviruses or retroviral vectors to create transgenic animals known to those skilled in the art involves microinjecting mitomycin C-treated cells or retroviral particles producing retrovirus into the perivitelline space of fertilized eggs or early embryos (see Haskell and Bowen (1995) *Mol. Reprod. Dev.* 40:386).

[0124] In one embodiment of the invention, non-human transgenic animals can be generated that express a modified A1 domain of the VWF sequence. The modified A1 domain can contain an amino acid residue substitution at a position involved with binding of the VWF protein to GPIb alpha (such as, but not limited to, positions 1263, 1269, 1274, 1287, 1302, 1308, 1313, 1314, 1326, 1329, 1330, 1333, 1344, 1347, 1350, 1370, 1379, 1381, 1385 1391, 1394, 1397, 1421, 1439, 1442, 1449, 1466, 1469, 1472, 1473, 1475, 1479). In some embodiments, the VWF-A1 domain can comprise a single mutation, while in other embodiments, it can comprise 2 or more mutations.

[0125] In one embodiment of the invention, the modification of the A1 domain can be a partial or full replacement of an animal (for example, a mouse) A1 domain of VWF with the A1 domain of human VWF. In other words, the A1 domain in the animal VWF is removed and replaced with the human A1 sequence. In another embodiment, the animal VWF A1 domain may be partially replaced so that some portion of the human A1 domain replaces a portion of the animal A1 domain. For example, human A1 domain sequence could comprises at least 5%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 90%, 95%, 99%, or 100% of the animal A1 domain. The A1 domain of human VWF protein comprises SEQ ID NO: 1. In another embodiment, the animal can be a model for pre-clinical testing of compounds, wherein the animal expresses a mutant von Willebrand Factor (VWF) A1 protein containing one or more mutations, such that the binding specificity of the mutant VWF-A1 protein changes from being specific for the animal platelets to being specific for human platelets. In another embodiment, the mutation occurs in the VWF-A1 domain of a mouse. In particular embodiments, the murine mutant VWF-A1 protein comprises at least one mutation comprising 1263P>S, 1269N>D, 1274K>R, 1287M>R, 1302G>D, 1308H>R, 1313R>W, 1314I>V, 1326R>H, 1329L>I, 1330E>G, 1333A>D, 1344T>A, 1347I>V, 1350T>A, 1370G>S, 1379H>R, 1381T>A, 1385T>M 1391P>Q, 1394A>S, 1397L>F, 1421S>N, 1439L>V, 1442G>S, 1449R>Q, 1466A>P, 1469Q>L, 1472Q>H, 1473V>M, 1475H>Q, 1479S>G, or any combination thereof. In a particularly useful embodiment, the murine mutant VWF-A1 protein comprises a 1326R>H mutation, a 1314I>V mutation, or a combination thereof.

[0126] The modification of the A1 domain can be an amino acid substitution at residue 1326 (for example Arg for His in the mouse). In some embodiments, the non-human transgenic animal harbors a mutant construct wherein an amino acid residue substitution at a position involved affects binding to GPIb alpha (such as, but not limited to, positions 1263, 1269, 1274, 1287, 1302, 1308, 1313, 1314, 1326, 1329, 1330, 1333, 1344, 1347, 1350, 1370, 1379, 1381, 1385 1391, 1394, 1397, 1421, 1439, 1442, 1449, 1466, 1469, 1472, 1473, 1475, 1479). In yet other embodiments, non-human transgenic animals can successfully harbor a type 2B (Ile1309Val; 1309I>V) mutation and/or an Arg1326His (1326R>H) mutant construct. In another embodiment, the non-human transgenic animal expresses an Arg1326His (1326R>H) mutation wherein the mutant VWF-A1 domain comprises SEQ ID NO: 5, which corresponds to the His amino acid at the same position in humans, canines, chimpanzees, rat, porcine, felines, equines, bovine, and the like (Jenkins et al., (1998) *Blood* 91(6): 2032-44). In further embodiments of the inven-

tion, the non-human transgenic animal is a mouse. Example 3 below describes the transgenic animal of the current invention.

[0127] Molecular Manipulations of VWF-A1 and its Corresponding Mutants

[0128] The present invention utilizes conventional molecular biology, microbiology, and recombinant DNA techniques available to one of ordinary skill in the art. Such techniques are well known to the skilled worker and are explained fully in the literature. See, e.g., Maniatis, Fritsch & Sambrook, "*Molecular Cloning: A Laboratory Manual*" (1982): "*DNA Cloning: A Practical Approach,*" Volumes I and II (D. N. Glover, ed., 1985); "Oligonucleotide Synthesis" (M. J. Gait, ed., 1984); "*Nucleic Acid Hybridization*" (B. D. Hames & S. J. Higgins, eds., 1985); "*Transcription and Translation*" (B. D. Hames & S. J. Higgins, eds., 1984); "*Animal Cell Culture*" (R. I. Freshney, ed., 1986); "*Immobilized Cells and Enzymes*" (IRL Press, 1986): B. Perbal, "*A Practical Guide to Molecular Cloning*" (1984), and Sambrook, et al., "*Molecular Cloning: a Laboratory Manual*" (1989).

[0129] The VWF sequences from mouse, human, have been aligned as shown in Jenkins et al. (1998) "Molecular Modeling of Ligand and Mutation Sites of the Type A Domains of Human von Willebrand Factor and Their Relevance to von Willebrand's Disease" Vol. 91, No. 6, *Blood, pp.* 2032-2044.

[0130] The DNA and polypeptide sequences of human VWF are readily available to those skilled in the art, under Genbank Accession No. X04385. The polypeptide sequence of the A1 domain of human VWF, which runs from amino acid residue number 1260 to amino acid residue number 1480 of the nucleotide sequence of SEQ ID NO:6, is shown in SEQ ID NO: 1. The polypeptide sequence of the A1 domain of mouse VWF, which runs from amino acid residue number 1260 to amino acid residue number 1480 of the of SEQ ID NO:8, is shown in SEQ ID NO: 2.

[0131] SEQ ID NO: 1 is the human wild type amino acid sequence corresponding to the A1 domain of VWF. The residues shown in SEQ ID NO: 1 are residues 1260-1480, the A1 domain, of SEQ ID NO: 6.

SEQ ID NO:1:
EDISEPPLHDFYCSRLLDLVFLLDGSSRLSEAEFEVLKAFVVDMMERLRI

SQKWVRVAVVEYHDGSHAYIGLKDRKRPSELRRIASQVKYAGSQVASTSE

VLKYTLFQIFSKIDRPEASRIALLLMASQEPQRMSRNFVRYVQGLKKKKV

IVIPVGIGPHANLKQIRLIEKQAPENKAFVLSSVDELEQQRDEIVSYLCD

LAPEAPPPTLPPHMAQVTVGP

[0132] SEQ ID NO: 2 is the mouse wild type amino acid sequence corresponding to the A1 domain of VWF. The residues shown in SEQ ID NO:2 are residue numbers 1260-1480 from the full length mouse VWF shown in SEQ ID NO: 8.

SEQ ID NO:2:
EDTPEPPLHNFYCSKLLDLVFLLDGSSMLSEAEFEVLKAFVVGMMERLHI

SQKRIRVAVVEYHDGSRAYLELKARKRPSELRRITSQIKYTGSQVASTSE

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VLKYTLFQIFGKIDRPEASHITLLLTASQEPPRMARNLVRYVQGLKKKKV

IVIPVGIGPHASLKQIRLIEKQAPENKAFLLSGVDELEQRRDEIVSYLCD

LAPEAPAPTQPPQVAHVTVSP

[0133] The nucleotide sequence of the A1 domain of human VWF corresponding to amino acid residues 1260-1480 is shown in SEQ ID NO: 3 and of mouse VWF is shown in SEQ ID NO: 4 below.

[0134] SEQ ID NO: 3 is the human wild type nucleotide sequence corresponding to the A1 domain of VWF:

GAGGACATCTCGGAACCGCCGTTGCACGATTTCTACTGCAGCAGGCTACT

GGACCTGGTCTTCCTGCTGGATGGCTCCTCCAGGCTGTCCGAGGCTGAGT

TTGAAGTGCTGAAGGCCTTTGTGGTGGACATGATGGAGCGGCTGCGCATC

TCCCAGAAGTGGGTCCGCGTGGCCGTGGTGGAGTACCACGACGGCTCCCA

CGCCTACATCGGGCTCAAGGACCGGAAGCGACCGTCAGAGCTGCGGCGCA

TTGCCAGCCAGGTGAAGTATGCGGGCAGCCAGGTGGCCTCCACCAGCGAG

GTCTTGAAATACACACTGTTCCAAATCTTCAGCAAGATCGACCGCCCTGA

AGCCTCCCGCATCGCCCTGCTCCTGATGGCCAGCCAGGAGCCCCAACGGA

TGTCCCGGAACTTTGTCCGCTACGTCCAGGGCCTGAAGAAGAAGAAGGTC

ATTGTGATCCCGGTGGGCATTGGGCCCCATGCCAACCTCAAGCAGATCCG

CCTCATCGAGAAGCAGGCCCCTGAGAACAAGGCCTTCGTGCTGAGCAGTG

TGGATGAGCTGGAGCAGCAAAGGGACGAGATCGTTAGCTACCTCTGTGAC

CTTGCCCCTGAAGCCCCTCCTCCTACTCTGCCCCCCCACATGGCACAAGT

CACTGTGGGCCCG

[0135] SEQ ID NO: 4 is the mouse wild type nucleotide sequence corresponding to the A1 domain of VWF:

GAGGATACCCCCGAGCCCCCCCTGCACAACTTCTACTGCAGCAAGCTGCT

GGATCTTGTCTTCCTGCTGGATGGCTCCTCTATGTTGTCCGAGGCTGAGT

TTGAAGTGCTCAAAGCTTTTGTGGTGGGCATGATGGAGAGGTTACACATC

TCTCAGAAGCGCATCCGCGTGGCAGTGGTAGAGTACCATGATGGCTCCCG

TGCCTACCTTGAGCTCAAGGCCCGGAAGCGACCCTCAGAGCTTCGGCGCA

TCACCAGCCAGATTAAGTATACAGGCAGCCAGGTGGCCTCTACCAGTGAG

GTTTTGAAGTACACACTGTTCCAGATCTTTGGCAAAATTGACCGCCCTGA

AGCCTCCCATATCACTCTGCTCCTGACTGCTAGCCAGGAGCCCCCACGGA

TGGCTAGGAATTTGGTCCGCTATGTCCAAGGTCTGAAGAAGAAGAAGGTT

ATCGTGATCCCTGTGGGCATTGGGCCCCACGCCAGCCTCAAACAGATCCG

CCTCATCGAGAAGCAGGCCCCTGAAAACAAGGCTTTTCTGCTCAGTGGGG

TGGATGAGCTGGAGCAGAGAAGAGATGAGATAGTCAGCTACCTCTGTGAC

CTTGCTCCCGAGGCCCCAGCCCCAACTCAGCCTCCACAGGTAGCCCACGT

CACCGTGAGTCCA

[0136] Human mRNA for pre-pro-von Willebrand factor:

SEQ ID NO: 6: Amino Acid Sequence Human VWF-(residue 1 to residue 2813)

```
   1 MIPARFAGVL LALALILPGT LCAEGTRGRS STARCSLFGS DFVNTFDGSM YSFAGYCSYL

  61 LAGGCQKRSF SIIGDFQNGK RVSLSVYLGE FFDIHLFVNG TVTQGDQRVS MPYASKGLYL

 121 ETEAGYYKLS GEAYGFVARI DGSGNFQVLL SDRYFNKTCG LCGNFNIFAE DDFMTQEGTL

 181 TSDPYDFANS WALSSGEQWC ERASPPSSSC NISSGEMQKG LWEQCQLLKS TSVFARCHPL

 241 VDPEPFVALC EKTLCECAGG LECACPALLE YARTCAQEGM VLYGWTDHSA CSPVCPAGME

 301 YRQCVSPCAR TCQSLHINEM CQERCVDGCS CPEGQLLDEG LCVESTECPC VHSGKRYPPG

 361 TSLSRDCNTC ICRNSQWICS NEECPGECLV TGQSHFKSFD NRYFTFSGIC QYLLARDCQD

 421 HSFSIVIETV QCADDRDAVC TRSVTVRLPG LHNSLVKLKH GAGVAMDGQD IQLPLLKGDL

 481 RIQHTVTASV RLSYGEDLQM DWDGRGRLLV KLSPVYAGKT CGLCGNYNGN QGDDFLTPSG

 541 LAEPRVEDFG NAWKLHGDCQ DLQKQHSDPC ALNPRMTRFS EEACAVLTSP TFEACHRAVS

 601 PLPYLRNCRY DVCSCSDGRE CLCGALASYA AACAGRGVRV AWREPGRCEL NCPKGQVYLQ

 661 CGTPCNLTCR SLSYPDEECN EACLEGCFCP PGLYMDERGD CVPKAQCPCY YDGEIFQPED

 721 IFSDHHTMCY CEDGFMHCTM SGVPGSLLPD AVLSSPLSHR SKRSLSCRPP MVKLVCPADN

 781 LRAEGLECTK TCQNYDLECM SMGCVSGCLC PPGMVRHENR CVALERCPCF HQGKEYAPGE

 841 TVKIGCNTCV CRDRKWNCTD HVCDATCSTI GMAHYLTFDG LKYLFPGECQ YVLVQDYCGS

 901 NPGTFRILVG NKGCSHPSVK CKKRVTILVE GGEIELFDGE VNVKRPMKDE THFEVVESGR

 961 YIILLLGKAL SVVWDRHLSI SVVLKQTYQE KVCGLCGNFD GIQNNDLTSS NLQVEEDPVD

1021 FGNSWKVSSQ CADTRKVPLD SSPATCHNNI MKQTMVDSSC RILTSDVFQD CNKLVDPEPY

1081 LDVCIYDTCS CESIGDCACF CDTIAAYAHV CAQHGKVVTW RTATLCPQSC EERNLRENGY

1141 ECEWRYNSCA PACQVTCQHP EPLACPVQCV EGCHAHCPPG KILDELLQTC VDPEDCPVCE

1201 VAGRRFASGK KVTLNPSDPE HCQICHCDVV NLTCEACQEP GGLVVPPTDA PVSPTTLYVE

1261 DISEPPLHDF YCSRLLDLVF LLDGSSRLSE AEFEVLKAFV VDMMERLRIS QKWVRVAVVE

1321 YHDGSHAYIG LKDRKRPSEL RRIASQVKYA GSQVASTSEV LKYTLFQIFS KIDRPEASRI

1381 ALLLMASQEP QRMSRNFVRY VQGLKKKKVI VIPVGIGPHA NLKQIRLIEK QAPENKAFVL

1441 SSVDELEQQR DEIVSYLCDL APEAPPPTLP PHMAQVTVGP GLLGVSTLGP KRNSMVLDVA

1501 FVLEGSDKIG EADFNRSKEF MEEVIQRMDV GQDSIHVTVL QYSYMVTVEY PFSEAQSKGD

1561 ILQRVREIRY QGGNRTNTGL ALRYLSDHSF LVSQGDREQA PNLVYMVTGN PASDEIKRLP

1621 GDIQVVPIGV GPNANVQELE RIGWPNAPIL IQDFETLPRE APDLVLQRCC SGEGLQIPTL

1681 SPAPDCSQPL DVILLLDGSS SFPASYFDEM KSFAKAFISK ANIGPRLTQV SVLQYGSITT

1741 IDVPWNVVPE KAHLLSLVDV MQREGGPSQI GDALGFAVRY LTSEMHGARP GASKAVVILV

1801 TDVSVDSVDA AADAARSNRV TVFPIGIGDR YDAAQLRILA GPAGDSNVVK LQRIEDLPTM

1861 VTLGNSFLHK LCSGFVRICM DEDGNEKRPG DVWTLPDQCH TVTCQPDGQT LLKSHRVNCD

1921 RGLRPSCPNS QSPVKVEETC GCRWTCPCVC TGSSTRHIVT FDGQNFKLTG SCSYVLFQNK

1981 EQDLEVILHN GACSPGARQG CMKSIEVKHS ALSVELHSDM EVTVNGRLVS VPYVGGNMEV

2041 NVYGAIMHEV RFNHLGHIFT FTPQNNEFQL QLSPKTFASK TYGLCGICDE NGANDFMLRD

2101 GTVTTDWKTL VQEWTVQRPG QTCQPILEEQ CLVPDSSHCQ VLLLPLFAEC HKVLAPATFY

2161 AICQQDSCHQ EQVCEVIASY AHLCRTNGVC VDWRTPDFCA MSCPPSLVYN HCEHGCPRHC
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2221 DGNVSSCGDH PSEGCFCPPD KVMLEGSCVP EEACTQCIGE DGVQHQFLEA WVPDHQPCQI

2281 CTCLSGRKVN CTTQPCPTAK APTCGLCEVA RLRQNADQCC PEYECVCDPV SCDLPPVPHC

2341 ERGLQPTLTN PGECRPNFTC ACRKEECKRV SPPSCPPHRL PTLRKTQCCD EYECACNCVN

2401 STVSCPLGYL ASTATNDCGC TTTTCLPDKV CVHRSTIYPV GQFWEEGCDV CTCTDMEDAV

2461 MGLRVAQCSQ KPCEDSCRSG FTYVLHEGEC CGRCLPSACE VVTGSPRGDS QSSWKSVGSQ

2521 WASPENPCLI NECVRVKEEV FIQQRNVSCP QLEVPVCPSG FQLSCKTSAC CPSCRCERME

2581 ACMLNGTVIG PGKTVMIDVC TTCRCMVQVG VISGFKLECR KTTCNPCPLG YKEENNTGEC

2641 CGRCLPTACT IQLRGGQIMT LKRDETLQDG CDTHFCKVNE RGEYFWEKRV TGCPPFDEHK

2701 CLAEGGKIMK IPGTCCDTCE EPECNDITAR LQYVKVGSCK SEVEVDIHYC QGKCASKAMY

2761 SIDINDVQDQ CSCCSPTRTE PMQVALHCTN GSVVYHEVLN AMECKCSPRK CSK

VWF mature peptide (AA 763-2790)

VWF pro polypeptide (AA 1-2790)

SEQ ID NO: 7 Nucleic acid sequence-human VWF

1 agctcacagc tattgtggtg ggaaagggag ggtggttggt ggatgtcaca gcttgggctt

61 tatctccccc agcagtgggg actccacagc ccctgggcta cataacagca agacagtccg

121 gagctgtagc agacctgatt gagcctttgc agcagctgag agcatggcct agggtgggcg

181 gcaccattgt ccagcagctg agtttcccag ggaccttgga gatagccgca gccctcattt

241 gcaggggaag gcaccattgt ccagcagctg agtttcccag ggaccttgga gatagccgca

301 gccctcattt atgattcctg ccagatttgc cggggtgctg cttgctctgg ccctcatttt

361 gccagggacc ctttgtgcag aaggaactcg cggcaggtca tccacggccc gatgcagcct

421 tttcggaagt gacttcgtca acacctttga tgggagcatg tacagctttg cgggatactg

481 cagttacctc ctggcagggg gctgccagaa acgctccttc tcgattattg gggacttcca

541 gaatggcaag agagtgagcc tctccgtgta tcttggggaa ttttttgaca tccatttgtt

601 tgtcaatggt accgtgacac agggggacca aagagtctcc atgccctatg cctccaaagg

661 gctgtatcta gaaactgagg ctgggtacta caagctgtcc ggtgaggcct atggctttgt

721 ggccaggatc gatggcagcg gcaactttca agtcctgctg tcagacagat acttcaacaa

781 gacctgcggg ctgtgtggca actttaacat ctttgctgaa gatgacttta tgacccaaga

841 agggaccttg acctcggacc cttatgactt tgccaactca tgggctctga gcagtggaga

901 acagtggtgt gaacgggcat ctcctcccag cagctcatgc aacatctcct ctggggaaat

961 gcagaagggc ctgtgggagc agtgccagct tctgaagagc acctcggtgt ttgcccgctg

1021 ccaccctctg gtggaccccg agccttttgt ggccctgtgt gagaagactt tgtgtgagtg

1081 tgctgggggg ctggagtgcg cctgccctgc cctcctggag tacgcccgga cctgtgccca

1141 ggagggaatg gtgctgtacg gctggaccga ccacagcgcg tgcagcccag tgtgccctgc

1201 tggtatggag tataggcagt gtgtgtcccc ttgcgccagg acctgccaga gcctgcacat

1261 caatgaaatg tgtcaggagc gatgcgtgga tggctgcagc tgccctgagg gacagctcct

1321 ggatgaaggc ctctgcgtgg agagcaccga gtgtccctgc gtgcattccg gaaagcgcta

1381 ccctcccggc acctccctct ctcgagactg caacacctgc atttgccgaa acagccagtg

1441 gatctgcagc aatgaagaat gtccagggga gtgccttgtc actggtcaat cccacttcaa

1501 gagctttgac aacagatact tcaccttcag tgggatctgc cagtacctgc tggcccggga

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1561 ttgccaggac cactccttct ccattgtcat tgagactgtc cagtgtgctg atgaccgcga

1621 cgctgtgtgc acccgctccg tcaccgtccg gctgcctggc ctgcacaaca gccttgtgaa

1681 actgaagcat ggggcaggag ttgccatgga tggccaggac atccagctcc ccctcctgaa

1741 aggtgacctc cgcatccagc atacagtgac ggcctccgtg cgcctcagct acggggagga

1801 cctgcagatg gactgggatg gccgcgggag gctgctggtg aagctgtccc ccgtctacgc

1861 cgggaagacc tgcggcctgt gtgggaatta caatggcaac cagggcgacg acttccttac

1921 cccctctggg ctggcagagc cccgggtgga ggacttcggg aacgcctgga agctgcacgg

1981 ggactgccag gacctgcaga agcagcacag cgatccctgc gccctcaacc cgcgcatgac

2041 caggttctcc gaggaggcgt gcgcggtcct gacgtccccc acattcgagg cctgccatcg

2101 tgccgtcagc ccgctgccct acctgcggaa ctgccgctac gacgtgtgct cctgctcgga

2161 cggccgcgag tgcctgtgcg gcgccctggc cagctatgcc gcggcctgcg cggggagagg

2221 cgtgcgcgtc gcgtggcgcg agccaggccg ctgtgagctg aactgcccga aaggccaggt

2281 gtacctgcag tgcgggaccc cctgcaacct gacctgccgc tctctctctt acccggatga

2341 ggaatgcaat gaggcctgcc tggagggctg cttctgcccc ccagggctct acatggatga

2401 gaggggggac tgcgtgccca aggcccagtg cccctgttac tatgacggtg agatcttcca

2461 gccagaagac atcttctcag accatcacac catgtgctac tgtgaggatg gcttcatgca

2521 ctgtaccatg agtggagtcc ccggaagctt gctgcctgac gctgtcctca gcagtccct

2581 gtctcatcgc agcaaaagga gcctatcctg tcggcccccc atggtcaagc tggtgtgtcc

2641 cgctgacaac ctgcgggctg aagggctcga gtgtaccaaa acgtgccaga actatgacct

2701 ggagtgcatg agcatgggct gtgtctctgg ctgcctctgc cccccgggca tggtccggca

2761 tgagaacaga tgtgtggccc tggaaaggtg tccctgcttc catcagggca aggagtatgc

2821 ccctggagaa acagtgaaga ttggctgcaa cacttgtgtc tgtcgggacc ggaagtggaa

2881 ctgcacagac catgtgtgtg atgccacgtg ctccacgatc ggcatggccc actacctcac

2941 cttcgacggg ctcaaatacc tgttccccgg ggagtgccag tacgttctgg tgcaggatta

3001 ctgcggcagt aaccctggga cctttcggat cctagtgggg aataagggat gcagccaccc

3061 ctcagtgaaa tgcaagaaac gggtcaccat cctggtggag ggaggagaga ttgagctgtt

3121 tgacggggag gtgaatgtga agaggcccat gaaggatgag actcactttg aggtggtgga

3181 gtctggccgg tacatcattc tgctgctggg caaagccctc tccgtggtct gggaccgcca

3241 cctgagcatc tccgtggtcc tgaagcagac ataccaggag aaagtgtgtg gcctgtgtgg

3301 gaattttgat ggcatccaga acaatgacct caccagcagc aacctccaag tggaggaaga

3361 ccctgtggac tttgggaact cctggaaagt gagctcgcag tgtgctgaca ccagaaaagt

3421 gcctctggac tcatcccctg ccacctgcca taacaacatc atgaagcaga cgatggtgga

3481 ttcctcctgt agaatcctta ccagtgacgt cttccaggac tgcaacaagc tggtggaccc

3541 cgagccatat ctggatgtct gcatttacga cacctgctcc tgtgagtcca ttggggactg

3601 cgcctgcttc tgcgacacca ttgctgccta tgcccacgtg tgtgcccagc atggcaaggt

3661 ggtgacctgg aggacggcca cattgtgccc ccagagctgc gaggagagga atctccggga

3721 gaacgggtat gagtgtgagt ggcgctataa cagctgtgca cctgcctgtc aagtcacgtg

3781 tcagcaccct gagccactgg cctgccctgt gcagtgtgtg gagggctgcc atgcccactg

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3841 ccctccaggg aaaatcctgg atgagctttt gcagacctgc gttgaccctg aagactgtcc

3901 agtgtgtgag gtggctggcc ggcgttttgc ctcaggaaag aaagtcacct tgaatcccag

3961 tgaccctgag cactgccaga tttgccactg tgatgttgtc aacctcacct gtgaagcctg

4021 ccaggagccg ggaggcctgg tggtgcctcc cacagatgcc ccggtgagcc ccaccactct

4081 gtatgtggag gacatctcgg aaccgccgtt gcacgatttc tactgcagca ggctactgga

4141 cctggtcttc ctgctggatg gctcctccag gctgtccgag gctgagtttg aagtgctgaa

4201 ggcctttgtg gtggacatga tggagcggct gcgcatctcc cagaagtggg tccgcgtggc

4261 cgtggtggag taccacgacg gctcccacgc ctacatcggg ctcaaggacc ggaagcgacc

4321 gtcagagctg cggcgcattg ccagccaggt gaagtatgcg ggcagccagg tggcctccac

4381 cagcgaggtc ttgaaataca cactgttcca aatcttcagc aagatcgacc gccctgaagc

4441 ctcccgcatc gccctgctcc tgatggccag ccaggagccc caacggatgt cccggaactt

4501 tgtccgctac gtccagggcc tgaagaagaa gaaggtcatt gtgatcccgg tgggcattgg

4561 gccccatgcc aacctcaagc agatccgcct catcgagaag caggcccctg agaacaaggc

4621 cttcgtgctg agcagtgtgg atgagctgga gcagcaaagg gacgagatcg ttagctacct

4681 ctgtgacctt gcccctgaag cccctcctcc tactctgccc ccccacatgg cacaagtcac

4741 tgtgggcccg gggctcttgg gggtttcgac cctggggccc aagaggaact ccatggttct

4801 ggatgtggcg ttcgtcctgg aaggatcgga caaaattggt gaagccgact tcaacaggag

4861 caaggagttc atggaggagg tgattcagcg gatggatgtg ggccaggaca gcatccacgt

4921 cacggtgctg cagtactcct acatggtgac cgtggagtac cccttcagcg aggcacagtc

4981 caaaggggac atcctgcagc gggtgcgaga gatccgctac cagggcggca acaggaccaa

5041 cactgggctg gccctgcggt acctctctga ccacagcttc ttggtcagcc agggtgaccg

5101 ggagcaggcg cccaacctgg tctacatggt caccggaaat cctgcctctg atgagatcaa

5161 gaggctgcct ggagacatcc aggtggtgcc cattggagtg ggccctaatg ccaacgtgca

5221 ggagctggag aggattggct ggcccaatgc ccctatcctc atccaggact ttgagacgct

5281 cccccgagag gctcctgacc tggtgctgca gaggtgctgc tccggagagg ggctgcagat

5341 ccccaccctc tcccctgcac ctgactgcag ccagcccctg gacgtgatcc ttctcctgga

5401 tggctcctcc agtttcccag cttcttattt tgatgaaatg aagagtttcg ccaaggcttt

5461 catttcaaaa gccaatatag ggcctcgtct cactcaggtg tcagtgctgc agtatggaag

5521 catcaccacc attgacgtgc catggaacgt ggtcccggag aaagcccatt tgctgagcct

5581 tgtggacgtc atgcagcggg agggaggccc cagccaaatc ggggatgcct tgggctttgc

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5701 catcctggtc acggacgtct ctgtggattc agtggatgca gcagctgatg ccgccaggtc

5761 caacagagtg acagtgttcc ctattggaat tggagatcgc tacgatgcag cccagctacg

5821 gatcttggca ggcccagcag gcgactccaa cgtggtgaag ctccagcgaa tcgaagacct

5881 ccctaccatg gtcaccttgg gcaattcctt cctccacaaa ctgtgctctg gatttgttag

5941 gatttgcatg gatgaggatg ggaatgagaa gaggcccggg gacgtctgga ccttgccaga

6001 ccagtgccac accgtgactt gccagccaga tggccagacc ttgctgaaga gtcatcgggt

6061 caactgtgac cgggggctga ggccttcgtg ccctaacagc cagtcccctg ttaaagtgga

6121 agagacctgt ggctgccgct ggacctgccc ctgcgtgtgc acaggcagct ccactcggca

-continued

6181 catcgtgacc tttgatgggc agaatttcaa gctgactggc agctgttctt atgtcctatt

6241 tcaaaacaag gagcaggacc tggaggtgat tctccataat ggtgcctgca gccctggagc

6301 aaggcagggc tgcatgaaat ccatcgaggt gaagcacagt gccctctccg tcgagctgca

6361 cagtgacatg gaggtgacgg tgaatgggag actggtctct gttccttacg tgggtgggaa

6421 catggaagtc aacgtttatg gtgccatcat gcatgaggtc agattcaatc accttggtca

6481 catcttcaca ttcactccac aaaacaatga gttccaactg cagctcagcc ccaagacttt

6541 tgcttcaaag acgtatggtc tgtgtgggat ctgtgatgag aacggagcca atgacttcat

6601 gctgagggat ggcacagtca ccacagactg gaaaacactt gttcaggaat ggactgtgca

6661 gcggccaggg cagacgtgcc agcccatcct ggaggagcag tgtcttgtcc ccgacagctc

6721 ccactgccag gtcctcctct taccactgtt tgctgaatgc cacaaggtcc tggctccagc

6781 cacattctat gccatctgcc agcaggacag ttgccaccag gagcaagtgt gtgaggtgat

6841 cgcctcttat gcccacctct gtcggaccaa cggggtctgc gttgactgga ggacacctga

6901 tttctgtgct atgtcatgcc caccatctct ggtctacaac cactgtgagc atggctgtcc

6961 ccggcactgt gatggcaacg tgagctcctg tggggaccat ccctccgaag gctgtttctg

7021 ccctccagat aaagtcatgt tggaaggcag ctgtgtccct gaagaggcct gcactcagtg

7081 cattggtgag gatggagtcc agcaccagtt cctggaagcc tgggtcccgg accaccagcc

7141 ctgtcagatc tgcacatgcc tcagcgggcg gaaggtcaac tgcacaacgc agccctgccc

7201 cacggccaaa gctcccacgt gtggcctgtg tgaagtagcc cgcctccgcc agaatgcaga

7261 ccagtgctgc cccgagtatg agtgtgtgtg tgacccagtg agctgtgacc tgcccccagt

7321 gcctcactgt gaacgtggcc tccagcccac actgaccaac cctggcgagt gcagacccaa

7381 cttcacctgc gcctgcagga aggaggagtg caaaagagtg tccccaccct cctgcccccc

7441 gcaccgtttg cccacccttc ggaagaccca gtgctgtgat gagtatgagt gtgcctgcaa

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Mus musculus strain CASA/RkJ VWF (Vwf) mRNA
SEQ ID NO: 8-Amino Acid Sequence of Mouse VWF (residue no. 1 to residue no. 2813)

1 MNPFRYEICL LVLALTWPGT LCTEKPRDRP STARCSLFGD DFINTFDETM YSFAGGCSYL

61 LAGDCQKRSF SILGNFQDGK RMSLSVYLGE FFDIHLFANG TVTQGDQSIS MPYASQGLYL

121 EREAGYYKLS SETFGFAARI DGNGNFQVLM SDRHFNKTCG LCGDFNIFAE DDFRTQEGTL

181 TSDPYDFANS WALSSEEQRC KRASPPSRNC ESSSGDMHQA MWEQCQLLKT ASVFARCHPL

241 VDPESFVALC EKILCTCATG PECACPVLLE YARTCAQEGM VLYGWTDHSA CRPACPAGME

301 YKECVSPCPR TCQSLSINEV CQQQCVDGCS CPEGELLDED RCVQSSDCPC VHAGKRYPPG

361 TSLSQDCNTC ICRNSLWICS NEECPGECLV TGQSHFKSFD NRYFTFSGIC QYLLARDCED

421 HTFSIVIETM QCADDPDAVC TRSVSVRLSA LHNSLVKLKH GGAVGIDGQD VQLPFLQGDL

481 RIQHTVMASV RLSYAEDLQM DWDGRGRLLV KLSPVYSGKT CGLCGNYNGN KGDDFLTPAG

541 LVEPLVVDFG NAWKLQGDCS DLRRQHSDPC SLNPRLTRFA EEACALLTSS KFEACHHAVS

601 PLPYLQNCRY DVCSCSDSRD CLCNAVANYA AECARKGVHI GWREPGFCAL GCPQGQVYLQ

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781 PRAQGLECAK TCQNYDLECM SLGCVSGCLC PPGMVRHENK CVALERCPCF HQGAEYAPGD

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961 YVILLLGQAL SVVWDHHLSI SVVLKHTYQE QVCGLCGNFD GIQNNDFTTS SLQVEEDPVN

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1081 LDICIYDTCS CESIGDCACF CDTIAAYAHV CAQHGQVVAW RTPTLCPQSC EEKNVRENGY

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1561 VLRHVREIRY QGGNRTNTGQ ALQYLSEHSF SPSQGDRVEA PNLVYMVTGN PASDEIKRLP

1621 GDIQVVPIGV GPHANMQELE RISRPIAPIF IRDFETLPRE APDLVLQTCC SKEGLQLPTL

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1921 HGPRPSCANS QSPVRVEETC GCRWTCPCVC TGSSTRHIVT FDGQNFKLTG SCSYVIFQNK

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2461 VGLRVVQCSQ RPCEDSCQPG FSYVLHEGEC CGRCLPSACK VVAGSLRGDS HSSWKSVGSR

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2701 CLAEGGKIVK IPGTCCDTCE EPDCKDITAK VQYIKVGDCK SQEEVDIHYC QGKCASKAVY

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SEQ ID NO: 9-Nucleic Acid Sequence of Mouse VWF

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8521 ggattcctac tgatacc
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[0137] DNA sequences or oligonucleotides having specific sequences can be synthesized chemically or isolated by one of several approaches established in art. The basic strategies for identifying, amplifying, and isolated desired DNA sequences as well as assembling them into larger DNA molecules containing the desired sequence domains in the desired order, are well known to those of ordinary skill in the art. See, e.g., Sambrook, et al., (1989) *Nature* November 16; 342(6247):224-5; Perbal, B. et al., (1983) *J Virol.* March; 45(3):925-40. DNA segments corresponding to all or a portion of the VWF sequence may be isolated individually using the polymerase chain reaction (M. A. Innis, et al., "PCR Protocols: A Guide To Methods and Applications," Academic Press, 1990). A complete sequence may be assembled from overlapping oligonucleotides prepared by standard methods and assembled into a complete coding sequence. See, e.g., Edge (1981), *Nature,* 292:756; Nambiar, et al. (1984), *Science,* 223:1299; Jay et al. (1984), *J. Biol. Chem.,* 259:6311.

[0138] The assembled nucleotide sequence can be cloned into a suitable vector or replicon and maintained in said carrier in a composition that is substantially free of vectors that do not contain the assembled sequence, thus providing a reservoir of the assembled sequence wherein the entire sequence can be extracted from the reservoir via excising it from DNA material with restriction enzymes or by PCR amplification. Those of ordinary skill in the art are familiar with numerous cloning vectors, and the selection of an appropriate cloning vector is a matter of choice. The construction of vectors containing desired nucleotide sequences linked by appropriate DNA sequences is accomplished by discussed above. These vectors may be constructed to contain additional DNA sequences, such as bacterial origins of replication to make shuttle vectors in order to shuttle between prokaryotic hosts and mammalian hosts.

[0139] Procedures for construction and expression of mutant proteins of defined sequence are well known in the art. A DNA sequence encoding a mutant form of VWF or a fragment thereof can be synthesized chemically or prepared from the wild-type sequence by one of several approaches, including primer extension, linker insertion and PCR (see, e.g., Sambrook, et al., (1989) *Nature* November 16; 342(6247):224-5; Maniatis, Fritsch & Sambrook, "Molecular Cloning: A Laboratory Manual" (1982): "DNA Cloning: A Practical Approach," Volumes I and II (D. N. Glover, ed., 1985); "Oligonucleotide Synthesis" (M. J. Gait, ed., 1984); "Nucleic Acid Hybridization" (B. D. Hames & S. J. Higgins, eds., 1985); "Transcription and Translation" (B. D. Hames & S. J. Higgins, eds., 1984); "Animal Cell Culture" (R. I. Freshney, ed., 1986); "Immobilized Cells and Enzymes" (IRL Press, 1986): B. Perbal, "A Practical Guide to Molecular Cloning" (1984), and Sambrook, et al., "Molecular Cloning: a Laboratory Manual" (1989)). Mutants can be prepared by these techniques having additions, deletions, and substitutions in the wild-type sequence (for example, the mouse VWF-A1 1326R>H mutant of SEQ ID NO: 5). To confirm that the mutant contains the desired changes, one skilled in the art can confirm the changes of interest via sequence-by-sequence analysis and/or by methods available to one skilled in the art.

[0140] In one embodiment, modification of the A1 domain can contain an amino acid residue substitution at a position involved with binding to GPIb alpha (such as, but not limited to, positions 1263, 1269, 1274, 1287, 1302, 1308, 1313, 1314, 1326, 1329, 1330, 1333, 1344, 1347, 1350, 1370, 1379, 1381, 1385 1391, 1394, 1397, 1421, 1439, 1442, 1449, 1466, 1469, 1472, 1473, 1475, 1479). In further embodiments, the modification of the A1 domain can be a partial or full replacement of an animal (such as a mouse) A1 domain of VWF with the A1 domain of human VWF. In other embodiments of the invention, the modification of the A1 domain can be an amino acid substitution at residue 1326 (for example HIS for ARG) such as depicted in SEQ ID NO: 5.

[0141] SEQ ID NO: 5 is the sequence for the mouse VWF-A1 1326 R>H mutant, wherein the modified amino acid sequence corresponds to the A1 domain of mouse VWF (residues 1260-1480) having an amino acid substitution at residue 1326 of HIS for ARG (in Bold):

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EDTPEPPLHNFYCSKLLDLVFLLDGSSMLSEAEFEVLKAFVVGMMERLHI

SQKRIRVAVVEYHDGSHAYLELKARKRPSELRRITSQIKYTGSQVASTSE

VLKYTLFQIFGKIDRPEASHITLLLTASQEPPRMARNLVRYVQGLKKKKV

IVIPVGIGPHASLKQIRLIEKQAPENKAFLLSGVDELEQRRDEIVSYLCD

LAPEAPAPTQPPQVAHVTVSP
```

[0142] An expression vector containing a nucleotide sequence encoding a protein of interest, such as a mutant VWF-A1 molecule described above, is transfected into a host cell, either eukaryotic (for example, yeast, mammalian, or insect cells) or prokaryotic, by conventional techniques well established in the art. Transfection techniques carried out depend on the host cell used. For example, mammalian cell transfection can be accomplished using lipofection, protoplast fusion, DEAE-dextran mediated transfection, $CaPO_4$ co-precipitation, electroporation, direct microinjection, as well as other methods known in the art which can comprise: scraping, direct uptake, osmotic or sucrose shock, lysozyme

fusion or erythrocyte fusion, indirect microinjection such as via erythrocyte-mediated techniques, and/or by subjecting host cells to electric currents. Some of the techniques mentioned above are also applicable to unicellular organisms, such as bacteria or yeast. As other techniques for introducing genetic information into host cells will be developed, the above-mentioned list of transfection methods is not considered to be exhaustive. The transfected cells are then cultured by conventional techniques to produce a mutant VWF-A1 molecule harboring at least one of the mutations previously described.

[0143] One skilled in the art understands that expression of desired protein products in prokaryotes is most often carried out in *E. coli* with vectors that contain constitutive or inducible promoters. Some non-limiting examples of bacterial cells for transformation include the bacterial cell line *E. coli* strains DH5α or MC1061/p3 (Invitrogen Corp., San Diego, Calif.), which can be transformed using standard procedures practiced in the art, and colonies can then be screened for the appropriate plasmid expression. Some *E. coli* expression vectors (also known in the art as fusion-vectors) are designed to add a number of amino acid residues, usually to the N-terminus of the expressed recombinant protein. Such fusion vectors can serve three functions: 1) to increase the solubility of the desired recombinant protein; 2) to increase expression of the recombinant protein of interest; and 3) to aid in recombinant protein purification by acting as a ligand in affinity purification. In some instances, vectors, which direct the expression of high levels of fusion protein products that are readily purified, may also be used. Some non-limiting examples of fusion expression vectors include pGEX, which fuse glutathione S-tranferase to desired protein; pcDNA 3.1V5-His A B & C (Invitrogen Corp, Carlsbad, Calif.) which fuse 6×-His to the recombinant proteins of interest; pMAL (New England Biolabs, MA) which fuse maltose E binding protein to the target recombinant protein; the *E. coli* expression vector pUR278 (Ruther et al., (1983) *EMBO* 12:1791), wherein the coding sequence may be ligated individually into the vector in frame with the lac Z coding region in order to generate a fusion protein; and pIN vectors (Inouye et al., (1985) *Nucleic Acids Res.* 13:3101-3109; Van Heeke et al., (1989) *J. Biol. Chem.* 24:5503-5509. Fusion proteins generated by the likes of the above-mentioned vectors are generally soluble and can be purified easily from lysed cells via adsorption and binding to matrix glutathione agarose beads subsequently followed by elution in the presence of free glutathione. For example, the pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target can be released from the GST moiety.

[0144] Other suitable cell lines, in addition to microorganisms such as bacteria (e.g., *E. coli* and *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing coding sequences for a mutant VWF-A1 molecule described above, may alternatively be used to produce the molecule of interest. Non-limiting examples include plant cell systems infected with recombinant virus expression vectors (for example, tobacco mosaic virus, TMV; cauliflower mosaic virus, CaMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing coding sequences for a mutant VWF-A1 molecule described above; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing coding sequences for a mutant VWF-A1 molecule described above; yeast (for example, *Saccharomyces* sp., *Pichia* sp.) transformed with recombinant yeast expression vectors containing coding sequences for a mutant VWF-A1 molecule described above; or mammalian cell lines harboring a vector that contains coding sequences for a mutant VWF-A1 molecule described above.

[0145] Mammalian cells (such as BHK cells, VERO cells, CHO cells and the like) can also contain an expression vector (for example, one that harbors a nucleotide sequence encoding a mutant VWF-A1 molecule described above) for expression of a desired product. Expression vectors containing such a nucleic acid sequence linked to at least one regulatory sequence in a manner that allows expression of the nucleotide sequence in a host cell can be introduced via methods known in the art, as described above. To those skilled in the art, regulatory sequences are well known and can be selected to direct the expression of a protein of interest in an appropriate host cell as described in Goeddel, *Gene Expression Technology* (1990) *Methods in Enzymology* 185, Academic Press, San Diego, Calif.). Regulatory sequences can comprise the following: enhancers, promoters, polyadenylation signals, and other expression control elements. Practitioners in the art understand that designing an expression vector can depend on factors, such as the choice of host cell to be transfected and/or the type and/or amount of desired protein to be expressed.

[0146] Animal or mammalian host cells capable of harboring, expressing, and secreting large quantities of a mutant VWF-A1 molecule (described above) of interest into the culture medium for subsequent isolation and/or purification include, but are not limited to, Chinese hamster ovary cells (CHO), such as CHO-K1 (ATCC CCL-61), DG44 (Chasin et al., (1986) *Som. Cell Molec. Genet,* 12:555-556; Kolkekar et al., (1997) *Biochemistry,* 36:10901-10909; and WO 01/92337 A2), dihydrofolate reductase negative CHO cells (CHO/dhfr-, Urlaub et al., (1980) *Proc. Natl. Acad. Sci. U.S.A.,* 77:4216), and dp12.CHO cells (U.S. Pat. No. 5,721,121); monkey kidney CV1 cells transformed by SV40 (COS cells, COS-7, ATCC CRL-1651); human embryonic kidney cells (e.g., 293 cells, or 293 cells subcloned for growth in suspension culture, Graham et al., (1977) *J. Gen. Virol.,* 36:59); baby hamster kidney cells (BHK, ATCC CCL-10); monkey kidney cells (CV1, ATCC CCL-70); African green monkey kidney cells (VERO-76, ATCC CRL-1587; VERO, ATCC CCL-81); mouse sertoli cells (TM4; Mather (1980) *Biol. Reprod.,* 23:243-251); human cervical carcinoma cells (HELA, ATCC CCL-2); canine kidney cells (MDCK, ATCC CCL-34); human lung cells (W138, ATCC CCL-75); human hepatoma cells (HEP-G2, HB 8065); mouse mammary tumor cells (MMT 060562, ATCC CCL-51); buffalo rat liver cells (BRL 3A, ATCC CRL-1442); TRI cells (Mather (1982) *Annals NY Acad. Sci.,* 383:44-68); MCR 5 cells; FS4 cells. A cell line transformed to produce a mutant VWF-A1 molecule described above can also be an immortalized mammalian cell line of lymphoid origin, which include but are not limited to, a myeloma, hybridoma, trioma or quadroma cell line. The cell line can also comprise a normal lymphoid cell, such as a B cell, which has been immortalized by transformation with a virus, such as the Epstein Barr virus (such as a myeloma cell line or a derivative thereof).

[0147] A host cell strain, which modulates the expression of the inserted sequences, or modifies and processes the nucleic acid in a specific fashion desired also may be chosen. Such modifications (for example, glycosylation and other post-translational modifications) and processing (for example, cleavage) of protein products may be important for

the function of the protein. Different host cell strains have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. As such, appropriate host systems or cell lines can be chosen to ensure the correct modification and processing of the foreign protein expressed, such as a mutant VWF-A1 molecule described above. Thus, eukaryotic host cells possessing the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Non-limiting examples of mammalian host cells include 3T3, W138, BT483, Hs578T, CHO, VERY, BHK, Hela, COS, BT2O, T47D, NS0 (a murine myeloma cell line that does not endogenously produce any immunoglobulin chains), CRL7O3O, MDCK, 293, HTB2, and HsS78Bst cells.

[0148] For protein recovery, isolation and/or purification, the cell culture medium or cell lysate is centrifuged to remove particulate cells and cell debris. The desired polypeptide molecule (for example, a mutant VWF-A1 protein) is isolated or purified away from contaminating soluble proteins and polypeptides by suitable purification techniques. Non-limiting purification methods for proteins include: separation or fractionation on immunoaffinity or ion-exchange columns; ethanol precipitation; reverse phase HPLC; chromatography on a resin, such as silica, or cation exchange resin, e.g., DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, e.g., Sephadex G-75, Sepharose; protein A sepharose chromatography for removal of immunoglobulin contaminants; and the like. Other additives, such as protease inhibitors (e.g., PMSF or proteinase K) can be used to inhibit proteolytic degradation during purification. Purification procedures that can select for carbohydrates can also be used, e.g., ion-exchange soft gel chromatography, or HPLC using cation- or anion-exchange resins, in which the more acidic fraction(s) is/are collected.

[0149] In one embodiment, the protein isolated is a mutant human von Willebrand Factor A1 protein comprising one or more of the following mutations: 1263S>P, 1269D>N, 1274R>K, 1287R>M, 1302D>G, 1308R>H, 1313W>R, 1314V>I, 1326H>R, 1329I>L, 1330G>E, 1333D>A, 1344A>T, 1347V>I, 1350A>T, 1370S>G, 1379R>H, 1381A>T, 1385M>T 1391Q>P, 1394S>A, 1397F>L, 1421N>S, 1439V>L, 1442S>G, 1449Q>R, 1466P>A, 1469L>Q, 1472H>Q, 1473M>V, 1475Q>H, or 1479G>S. In another embodiment, the protein isolated is a mutant human von Willebrand Factor A1 protein comprising a 1263S>P, 1269D>N, 1274R>K, 1287R>M, 1302D>G, 1308R>H, 1313W>R, 1314V>I, 1326H>R, 1329I>L, 1330G>E, 1333D>A, 1344A>T, 1347V>I, 1350A>T, 1370S>G, 1379R>H, 1381A>T, 1385M>T, 1391Q>P, 1394S>A, 1397F>L, 1421N>S, 1439V>L, 1442S>G, 1449Q>R, 1466P>A, 1469L>Q, 1472H>Q, 1473M>V, 1475Q>H, or a 1479G>S mutation. In a particular embodiment, the protein isolated is a mutant human von Willebrand Factor A1 protein comprising a 1326H>R mutation.

[0150] The invention also provides a method for producing mutant von Willebrand Factor A1 protein that specifically binds human platelets. For example, an animal expressing a mutant von Willebrand Factor A1 (VWF-A1) protein can be provided, wherein the mutation causes the platelet binding specificity of the animal VWF-A1 protein to change to be specific for human platelets. VWF plasma protein containing the mutant A1 domain from an animal (such as from a mouse) can then be subsequently harvested. In one embodiment, the animal von Willebrand Factor A1 protein contains at least one mutation at amino acid position 1263, 1269, 1274, 1287, 1302, 1308, 1313, 1314, 1326, 1329, 1330, 1333, 1344, 1347, 1350, 1370, 1379, 1381, 1385, 1391, 1394, 1397, 1421, 1439, 1442, 1449, 1466, 1469, 1472, 1473, 1475, or 1479. In another embodiment, the mutations occur in a murine VWF-A1 protein. In particular embodiments, the mutant murine von Willebrand Factor A1 protein comprises at least one mutation comprising 1263P>S, 1269N>D, 1274K>R, 1287M>R, 1302G>D, 1308H>R, 1313R>W, 1314I>V, 1326R>H, 1329L>I, 1330E>G, 1333A>D, 1344T>A, 1347I>V, 1350T>A, 1370G>S, 1379H>R, 1381T>A, 1385T>M 1391P>Q, 1394A>S, 1397L>F, 1421S>N, 1439L>V, 1442G>S, 1449R>Q, 1466A>P, 1469Q>L, 1472Q>H, 1473V>M, 1475H>Q, 1479S>G, or any combination thereof.

[0151] Pre-Screening Evaluation of Anti-Thrombotics and Associated Diseases

[0152] Diversity libraries, such as random or combinatorial peptide or non-peptide libraries can be screened for small molecules and compounds that specifically bind to a VWF-A1 protein. Many libraries are known in the art that can be used such as, e.g., chemically synthesized libraries, recombinant (e.g., phage display) libraries, and in vitro translation-based libraries.

[0153] Any screening technique known in the art can be used to screen for agonist (i.e., compounds that promote platelet adhesion) or antagonist molecules (such as anti-thrombotics) directed at a target of interest (e.g. VWF-A1). The present invention contemplates screens for small molecule ligands or ligand analogs and mimics, as well as screens for natural ligands that bind to and modulate VWF-A1 binding to GPIb-alpha, such as via examining the degree of thrombus formation, platelet adhesion, coagulation, blood flow, vessel occlusion, or bleeding times. For example, natural products libraries can be screened using assays of the invention for molecules that modulate the activity of a molecule of interest, such as a VWF-A1 binding to GPIb-alpha.

[0154] Knowledge of the primary sequence of a molecule of interest, such as a VWF-A1, can provide an initial clue as to proteins that can modulate VWF-A1 binding to GPIb-alpha. Identification and screening of modulators is further facilitated by determining structural features of the protein, e.g., using X-ray crystallography, neutron diffraction, nuclear magnetic resonance spectrometry, and other techniques for structure determination. These techniques provide for the rational design or identification of such modulators.

[0155] Test compounds, such as test modulators of VWF-A1 binding to GPIb-alpha, are screened from large libraries of synthetic or natural compounds. Numerous means are currently used for random and directed synthesis of saccharide, peptide, and nucleic acid based compounds. Synthetic compound libraries are commercially available from Maybridge Chemical Co. (Trevillet, Cornwall, UK), Comgenex (Princeton, N.J.), Brandon Associates (Merrimack, N.H.), and Microsource (New Milford, Conn.). A rare chemical library is available from Aldrich (Milwaukee, Wis.). Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available from e.g. Pan Laboratories (Bothell, Wash.) or MycoSearch (N.C.), or are readily producible. Additionally, natural and synthetically produced libraries and compounds are readily modified through conventional chemical, physical, and biochemical means (Blondelle et al., (1996) *Tib Tech* 14:60).

[0156] Methods for preparing libraries of molecules are well known in the art and many libraries are commercially available. Libraries of interest in the invention include peptide libraries, randomized oligonucleotide libraries, synthetic organic combinatorial libraries, and the like. Degenerate peptide libraries can be readily prepared in solution, in immobilized form as bacterial flagella peptide display libraries or as phage display libraries. Peptide ligands can be selected from combinatorial libraries of peptides containing at least one amino acid. Libraries can be synthesized of peptoids and non-peptide synthetic moieties. Such libraries can further be synthesized which contain non-peptide synthetic moieties, which are less subject to enzymatic degradation compared to their naturally-occurring counterparts. Libraries are also meant to include for example but are not limited to peptide-on-plasmid libraries, polysome libraries, aptamer libraries, synthetic peptide libraries, synthetic small molecule libraries and chemical libraries. The libraries can also comprise cyclic carbon or heterocyclic structure and/or aromatic or polyaromatic structures substituted with one or more of the above-identified functional groups. Screening compound libraries listed above [also see EXAMPLE 6 and U.S. Patent Application Publication No. 2005/0009163, which is hereby incorporated by reference], in combination with dynamic force microscopy, a coagulation factor assay, a platelet adhesion assay, thrombus imaging, a bleeding time assay, aggregometry, review of real-time video of blood flow, a Doppler ultrasound vessel occlusion assay, or a combination of these assays (for example, those assays described in EXAMPLES 1-4) can be used to identify modulators of VWF-A1 binding to GPIb-alpha, wherein the compound abbreviates or increases off-rate (k_{off}) binding kinetics between VWF-A1 and GPIb-alpha by at least two-fold (Lew et al., (2000) *Curr. Med. Chem.* 7(6):663-72; Werner et al., (2006) *Brief Funct. Genomic Proteomic* 5(1):32-6).

[0157] Small molecule combinatorial libraries may also be generated. A combinatorial library of small organic compounds is a collection of closely related analogs that differ from each other in one or more points of diversity and are synthesized by organic techniques using multi-step processes. Combinatorial libraries include a vast number of small organic compounds. One type of combinatorial library is prepared by means of parallel synthesis methods to produce a compound array. A compound array can be a collection of compounds identifiable by their spatial addresses in Cartesian coordinates and arranged such that each compound has a common molecular core and one or more variable structural diversity elements. The compounds in such a compound array are produced in parallel in separate reaction vessels, with each compound identified and tracked by its spatial address. Examples of parallel synthesis mixtures and parallel synthesis methods are provided in U.S. Ser. No. 08/177,497, filed Jan. 5, 1994 and its corresponding PCT published patent application WO95/18972, published Jul. 13, 1995 and U.S. Pat. No. 5,712,171 granted Jan. 27, 1998 and its corresponding PCT published patent application WO96/22529, which are hereby incorporated by reference.

[0158] Examples of chemically synthesized libraries are described in Fodor et al., (1991) *Science* 251:767-773; Houghten et al., (1991) *Nature* 354:84-86; Lam et al., (1991) *Nature* 354:82-84; Medynski, (1994) *BioTechnology* 12:709-710; Gallop et al., (1994) *J. Medicinal Chemistry* 37(9):1233-1251; Ohlmeyer et al., (1993) *Proc. Natl. Acad. Sci. USA* 90:10922-10926; Erb et al., (1994) *Proc. Natl. Acad. Sci. USA* 91:11422-11426; Houghten et al., (1992) *Biotechniques* 13:412; Jayawickreme et al., (1994) *Proc. Natl. Acad. Sci. USA* 91:1614-1618; Salmon et al., (1993) *Proc. Natl. Acad. Sci. USA* 90:11708-11712; PCT Publication No. WO 93/20242, dated Oct. 14, 1993; and Brenner et al., (1992) *Proc. Natl. Acad. Sci. USA* 89:5381-5383.

[0159] Screening methods of the invention allowed for the identification of potential compounds that modulate VWF-A1 binding to GPIb-alpha. In some embodiments of the invention, the compound comprises one or more compounds having a structure depicted in Table 8 below.

[0160] Examples of phage display libraries are described in Scott et al., (1990) *Science* 249:386-390; Devlin et al., (1990) *Science,* 249:404-406; Christian, et al., (1992) *J. Mol. Biol.* 227:711-718; Lenstra, (1992) *J. Immunol. Meth.* 152:149-157; Kay et al., (1993) *Gene* 128:59-65; and PCT Publication No. WO 94/18318.

[0161] In vitro translation-based libraries include but are not limited to those described in PCT Publication No. WO 91/05058; and Mattheakis et al., (1994) *Proc. Natl. Acad. Sci. USA* 91:9022-9026.

[0162] In one non-limiting example, non-peptide libraries, such as a benzodiazepine library (see e.g., Bunin et al., (1994) *Proc. Natl. Acad. Sci. USA* 91:4708-4712), can be screened. Peptoid libraries, such as that described by Simon et al., (1992) *Proc. Natl. Acad. Sci. USA* 89:9367-9371, can also be used. Another example of a library that can be used, in which the amide functionalities in peptides have been permethylated to generate a chemically transformed combinatorial library, is described by Ostresh et al. (1994), *Proc. Natl. Acad. Sci. USA* 91:11138-11142.

[0163] Screening the libraries can be accomplished by any variety of commonly known methods. See, for example, the following references, which disclose screening of peptide libraries: Parmley and Smith, (1989) *Adv. Exp. Med. Biol.* 251:215-218; Scott and Smith, (1990) *Science* 249:386-390; Fowlkes et al., (1992) *BioTechniques* 13:422-427; Oldenburg et al., (1992) *Proc. Natl. Acad. Sci. USA* 89:5393-5397; Yu et al., (1994) *Cell* 76:933-945; Staudt et al., (1988) *Science* 241:577-580; Bock et al., (1992) *Nature* 355:564-566; Tuerk et al., (1992) *Proc. Natl. Acad. Sci. USA* 89:6988-6992; Ellington et al., (1992) *Nature* 355:850-852; U.S. Pat. Nos. 5,096,815; 5,223,409; and 5,198,346, all to Ladner et al.; Rebar et al., (1993) *Science* 263:671-673; and PCT Pub. WO 94/18318.

[0164] The invention provides a method for identifying a compound that modulates VWF-A1 binding to GPIb-alpha. In one embodiment, the method can comprise providing an electronic library of test compounds stored on a computer (such as those libraries described above); providing atomic coordinates for at least 10 amino acid residues of the A1 domain of the VWF protein listed in Table 8, where the coordinates having a root mean square deviation therefrom, with respect to at least 50% of the Cα atoms, of not greater than about 2.5 Å, in a computer readable format; converting the atomic coordinates into electrical signals readable by a computer processor to generate a three dimensional model of the VWF-A1 domain; performing a data processing method, wherein electronic test compounds from the library are superimposed upon the three dimensional model of the A1 domain of VWF; and determining which test compound fits within the binding pocket of the three dimensional model of the VWF-A1 protein. Thus, compounds can be identified that would modulate the binding of VWF-A1 to GPIb-alpha.

[0165] In another embodiment, the method can comprise providing an electronic library of test compounds stored on a computer; and providing atomic coordinates listed in Table 8 in a computer readable format for at least 10, 15, 20, 25, 30, 35, or 40 amino acid residues of the A1 domain of the VWF protein, wherein the residues can comprise 2 or more of the following residues: Pro1391, Arg1392, Arg1395, Val1398, Arg1399, Gln1402, Lys1406, Lys1423, Gln1424, Leu1427, Lys1430, or Glu1431; converting the atomic coordinates into electrical signals readable by a computer processor to generate a three dimensional model of the A1 domain of the VWF protein; performing a data processing method, wherein electronic test compounds from the library are superimposed upon the three dimensional model of the A1 domain of the VWF protein; and determining which test compound fits within the binding pocket of the three dimensional model of the VWF-A1 protein. Thus, compounds can be identified that would modulate the binding of VWF-A1 to GPIb-alpha.

[0166] In a further embodiment, the method can comprise providing an electronic library of test compounds stored on a computer (such as those libraries described above); providing atomic coordinates for at least 10 amino acid residues of the Botrocetin-VWF-A1 complex listed in accession entry 1IJK (http://www.rcsb.org/pdb/explore.do?structureId=1IJK), where coordinates having a root mean square deviation therefrom, with respect to at least 50% of the Cα atoms, not more than about 3 Å, in a computer readable format; converting the atomic coordinates into electrical signals readable by a computer processor to generate a three dimensional model of the Botrocetin-VWF-A1 complex; performing a data processing method, wherein electronic test compounds from the library are superimposed upon Botrocetin within the three dimensional model of the Botrocetin-VWF-A1 complex; and determining which test compound fits within the binding pocket of the three dimensional model of the VWF-A1 protein and best overlays the three-dimensional model generated above. Thus, compounds can be identified that would modulate the binding of VWF-A1 to GPIb-alpha.

[0167] In other embodiments, the method can comprise providing an electronic library of test compounds stored on a computer; providing atomic coordinates listed in accession entry 1IJK (http://www.rcsb.org/pdb/explore.do?structureId=1IJK) in a computer readable format for at least 10, 15, 20, 25, 30, 35, or 40 amino acid residues of the Botrocetin-murine VWF-A1 complex, wherein the residues comprise 2 or more of the following residues: Pro 1391, Arg1392, Arg1395, Val1398, Arg1399, Gln1402, Lys1406, Lys1423, Gln1424, Leu1427, Lys1430, or Glu1431; converting the atomic coordinates into electrical signals readable by a computer processor to generate a three dimensional model of the Botrocetin-VWF-A1 complex; performing a data processing method, wherein electronic test compounds from the library are superimposed upon Botrocetin within the three dimensional model of the Botrocetin-VWF-A1 complex; and determining which test compound fits within the binding pocket of the three dimensional model of the VWF-A1 protein and best overlays the three-dimensional model generated above. Thus, compounds can be identified that would modulate the binding of VWF-A1 to GPIb-alpha.

[0168] The invention also provides for a compound identified by the method described above. In one embodiment, the compound inhibits thrombosis formation or promotes platelet adhesion.

[0169] The present invention provides methods for evaluating potential anti-thrombotic reagents in pre-clinical testing using a non-human transgenic animal (for example, non-human animals include, but are not limited to, vertebrates such as ovines, bovines, rodents, non-human primates, porcines, caprines, equines, ruminants, lagomorphs, canines, felines, aves, and the like). There are three main classes of antithrombotic drugs that can be screened using the transgenic mouse model of the invention: Anticoagulant drugs (such as Heparins; Vitamin K antagonists, which are currently the only anticoagulants that can be administered orally; and direct thrombin inhibitors), Antiplatelet drugs (such as cyclooxygenase inhibitors like aspirin; phosphodiesterase inhibitors like ticlopidine (Ticlid); adenosine diphosphate receptor inhibitors like clopidogrel (Plavix); tirofiban (Aggrastat); adenosine reuptake inhibitors, and inhibitors of integrins on platelets (for example, alpha IIb Beta3) like eptifibatide (Integrilin)), and Thrombolytic or fibrinolytic drugs (such as t-PA (alteplase Activase); reteplase (Retavase); urokinase (Abbokinase); streptokinase (Kabikinase, Streptase); tenectaplase; lanoteplase; and anistreplase (Eminase)).

[0170] The invention provides an in vivo model to test the efficacy of potential anti-thrombotic drugs against human platelets prior to FDA approval. To date, in vitro models of thrombosis do not accurately recapitulate the hemodynamic conditions, cell-cell interactions, or cell-protein interactions that occur at sites of vascular injury in a living animal. Thus, anti-thrombotics can be identified and their potential therapeutic effects can be assessed for treatment of abnormal thrombotic events associated with atherothrombotic arterial diseases and venous thrombotic diseases (such as abnormal bleeding and/or abnormal clotting).

[0171] Atherothrombotic arterial diseases can include, but is not limited to, coronary artery disease, (for example, acute myocardial infarction, acute coronary syndromes (such as unstable angina pectoris) and stable angina pectoris); mesenteric ischemia, "abdominal angina," and mesenteric infarction; cerebral vascular disease, including acute stroke and transient ischemic attack; mesenteric arterial disease; as well as peripheral arterial disease, including acute peripheral arterial occlusion and intermittent claudication. Anti-thrombotic compounds identified by the pre-clinical testing method of the present invention can also be useful for the treatment of coronary artery disease (which includes, but is not limited to anti-thrombotic therapy during coronary angioplasty, anti-thrombotic therapy during cardiopulmonary bypass, and limiting of platelet activation during ischemia reperfusion) as well as venous thrombotic diseases (which include, but are not limited to deep venous thrombosis and pulmonary thromboembolism). Anti-thrombotic compounds identified by the pre-clinical testing method of the present invention can also be useful in anti-thrombotic therapy for pulmonary hypertension.

[0172] The invention provides a method for testing a compound that modulates VWF-A1 binding to GPIb-alpha. The method can entail obtaining or synthesizing a compound identified in the screens previously described above; contacting VWF-A1 with the compound under a condition suitable for GPIb-alpha-VWF-A1 binding; and determining whether the compound can modulate GPIb-alpha-VWF-A1 binding using a diagnostic assay. In one embodiment, contacting can comprises perfusing platelets into a flow chamber at a shear flow rate of at least 100 s^{-1}, wherein mutant murine VWF-A1

protein is immobilized on a bottom surface of the chamber. In another embodiment, contacting can comprise perfusing platelets into a transgenic non-human, for example the transgenic mouse described in EXAMPLE 3. In some embodiments, of the invention, contacting first occurs in vitro by way of the flow chamber described above, subsequently followed by in vivo testing of the compound's efficiency to modulate GPIb-alpha binding to VWF-A1 after the compound was determined to have a purported effect in modulating such binding in vitro. Thus, the invention provides a great advantage of being able to test directly compounds that target human platelets in an in vivo system. The transgenic mouse (which, for example, can harbor the 1326R>H mutation in the A1 domain of VWF of SEQ ID NO: 5) displays a bleeding phenotype, thus serves as a model for screening potential anti-thrombotic compounds useful for humans when the mouse is perfused with human platelets. Since the 1326R>H mutation in VWF-A1 in the mouse model (SEQ ID NO: 5) has been shown to support human platelet binding and it corresponds to the His amino acid at the same position in human VWF-A1 (as well as in canines, chimpanzees, rat, porcine, felines, equines, bovine, and the like (Jenkins et al., (1998) Blood 91(6): 2032-44)), the test compounds screened using this mouse model (while subject to perfusion with platelets from human, dog, cat, or other relevant organism) will be applicable to multiple species.

[0173] After perfusion with human platelets, a test compound (such as a purported anti-thrombotic that would minimize blood clotting or a compound that could promote platelet adhesion) can be administered to the animal subsequent to vessel injury in order to determine whether blood clotting is minimized or if it is enhanced. In one embodiment, the platelets infused are human platelets while in other embodiments the platelets infused are not murine platelets. In some embodiments, the compound can slow the on-rate, and/or increase the off-rate (k_{off}) binding kinetics, and/or reduce bond strength of the interaction between VWF-A1 and GPIb-alpha by at least two-fold, thus resulting in a decreased lifetime of the bond(s). Such compounds could reduce thrombosis formation. In other embodiments, the compound can abbreviate off-rate (k_{off}) binding kinetics between VWF-A1 and GPIb-alpha by at least two-fold, thus resulting in a prolongation in the lifetime of the bond(s). Such compounds could promote platelet adhesion due to the compound stabilizing an interaction between VWF-A1 and GPIb-alpha. To assess binding efficiency between VWF-A1 and GPIb-alpha, binding kinetics can be determined by measuring translocation velocity, tethering frequency, and bond strength (Fukuda, K., et al., (2005) *Nat. Struct. Mol. Biol.* 12:152-159; Doggett, et al., (2003) *Blood* 102(10): 152-60; Doggett, T. A. et al. (2002) *Biophys. J.* 83, 194-205; Schmidtke and Diamond (2000) *J Cell Bio* 149(3): 719-29; Mody et al., (2005) *Biophys. J.* 88: 1432-43, all of which are incorporated by reference in their entirety).

[0174] The compound identified and tested using the methods described above can be an anti-platelet drug. In one embodiment, the anti-platelet drug can be a cyclooxygenase inhibitor, a phosphodiesterase inhibitor, an adenosine diphosphate receptor inhibitor, a PI3K inhibitor, an adenosine reuptake inhibitors, thrombin receptor inhibitor or inhibitor of any intracellular signaling pathway in platelets, an alphaIIb beta3 inhibitor, an alpha2 beta1 inhibitor, a glycoprotein V inhibitor, a glycoprotein VI inhibitor, a PECAM-1 inhibitor or any adhesion molecule and/or activation pathway critical for human platelet function

[0175] The diagnostic assay used in this method for testing a compound that modulates VWF-A1 binding to GPIb-alpha can assess whether an abnormal thrombotic event occurred in the subject. An abnormal thrombotic event can comprise abnormal bleeding, abnormal clotting, death, or a combination thereof. The assay can comprise dynamic force microscopy, a coagulation factor assay, a platelet adhesion assay, thrombus imaging, a bleeding time assay, aggregometry, review of real-time video of blood flow, a Doppler ultrasound vessel occlusion assay, or a combination thereof.

[0176] After perfusion with a purported anti-thrombotic or a compound that could promote platelet adhesion, a labeled agent can subsequently be perfused either into the flow chamber or to the animal. Such an agent would enable the visualization of either the presence or absence of a thrombus. In one embodiment, the labeled agent can comprise one or more of a nanoparticle, a fluorophore, a quantum dot, a microcrystal, a radiolabel, a dye, a gold biolabel, an antibody, or a small molecule ligand. In some embodiments, the agent can target a platelet receptor, a VWF protein, or a portion thereof.

[0177] Methods for Assessing Thrombotic Events In Vivo

[0178] The invention provides methods for detecting an internal vascular injury site (occult bleeding) in a subject. This could be useful in ER settings or on the battlefield in order to quickly identify sites of internal bleeding. For instance, the method can entail: administering to a subject a targeted molecular imaging agent, wherein the molecule circulates for an effective period of time in order to bind to the injury site within the subject; tracking a deposition of the labeled thrombosis-indicating-molecule in the subject; and identifying the site of a thrombus formation in the subject by imaging the labeled targeted molecular imaging agent. Thus, the deposition of the targeted molecular imaging agent at the internal vascular injury site can be indicative of internal bleeding within a subject. For example, a targeted molecular imaging agent can recognize constituents of thrombi that comprise a lipid, a protein, a cellular molecule, or a combination thereof. In one embodiment, the targeted molecular imaging agent is administered by subcutaneous, intra-muscular, intra-peritoneal, or intravenous injection; infusion; by oral, nasal, or topical delivery; or a combination of the listed routes of administration. In other embodiments, the targeted molecular imaging agent has a $T_{1/2}$ of at least 30 min. In some embodiments, the targeted molecular imaging agent can comprise an antibody, peptide, or Fab fragment directed to a platelet receptor, a VWF protein, or a portion thereof. In particular embodiments, the targeted molecular imaging agent can comprise a VWF-A1 or GPIb-alpha receptor trap. For example, a receptor trap is a decoy receptor that can comprise fusions between two distinct receptor components and the Fc region of an antibody molecule, which can result in the generation of a molecule with an increased affinity over single component reagents. This technology is available from Regeneron (Tarrytown, N.Y.) and is described in Wachsberger et al., (2007) *Int J Radiat Oncol Biol Phys.* 67(5):1526-37; Holash et al., (2002) *Proc Natl Acad Sci U S A.* 2002 99(17):11393-8; Davis et al., (1996) *Cell.* 87(7):1161-9; U.S. Pat. No. 7,087,411; and in United States Publication Applications 2004/0014667, 2005/0175610, 2005/0260203, 2006/0030529, 2006/0058234, which are all hereby incorporated by reference in their entirety.

[0179] To aid in the visualization of a site of thrombus formation, the targeted molecular imaging agent can further comprise a label. In one embodiment, the labeled thrombosis-indicating-molecule comprises a nanoparticle, a fluorophore, a quantum dot, a microcrystal, a radiolabel, a dye, a gold biolabel, an antibody, a peptide, a small molecule ligand, or a combination thereof. A fluorophore, for example green fluorescent protein, (such as GFP, RFP, YFP and the like; see Johnson and Johnson, (2007) *ACS Chem. Biol.* 2(1):31-8) can be used as a biomarker. A quantum dot is a semiconductor nanocrystal, that can be as small as 2 to 10 nm or can 15-20 nm (for example, Q-dot nanocrystals; also see Kaji et al., (2007) *Anal Sci.* 23(1):21-4). Quantum dot fluorescence can be induced by exposure to ultraviolet light. Both a fluorescent protein and a quantum dot can be obtained commercially (for example, Molecular Probes—Invitrogen, Carlsbad, Calif. or Evident Technologies, Troy N.Y.). A fluorophore can also be generated in the laboratory according to molecular biology methods practiced in the art. A radiolabel is a radioactive isotope that can be used as a tracer. Non-limiting examples of radiolabels include: Technetium-99m, Iodine-123 and 131, Thallium-201, Gallium-67, Fluorine-18, -19, Indium-111, Xenon-I 33, and Krypton-81m. Radiolabels can be obtained commercially, for example, from SRI International (Menlo Park, Calif.). In one embodiment, the nanoparticle can comprise a perfluorocarbon (PFC). Non-limiting examples of perfluorocarbons include perfluorobutane, perfluorohexane, perfluorooctane, perfluorodecalin, perfluoromethyldecalin, and perfluoroperhydrophenanthrene. These can be synthesized according to the method described in EXAMPLE 5 or according to Partlow et al. (*FASEB J* (2007) February 6 on-line publication, fj.06-6505com). The perfluorocarbon molecules can also be obtained commercially (F2 Chemicals Ltd.; Lancashire, UK). In another embodiment, the PFC nanoparticle can be coupled to a platelet receptor antibody (such as platelet receptor alpha-IIb beta$_3$). In some embodiments, imaging can comprise a PET scan, a CT scan, an MRI, an IR scan, an ultrasound, nuclear imaging, or a combination thereof.

[0180] Since the usefulness of the method pertains to the swiftness in identifying sites of internal bleeding (for example in an ER setting or on the battlefield), the subject can be further administered a compound aid in the cessation of such bleeding. In one embodiment, the subject is further administered a thrombotic compound (for example, a compound identified in the screens described above or a compound comprising a structure depicted in Table 8). In some embodiments, the compound can abbreviate off-rate (k_{off}) binding kinetics, and/or slow the on-rate, and/or reduce the bond strength between VWF-A1 and GPIb-alpha by at least two-fold.

[0181] The invention also provides a method to test contrast agents for imaging of human platelets at sites of thrombosis. For instance, one could test the ability of nanoparticle contrast agents targeted to human platelets to identify areas of thrombosis or occult bleeding. In some embodiments, the prevention or reduction of thrombus formation at site of injury upon administration of a compound can be visually examined via tracking the localization of labeled platelets (such as with high resolution in vivo microscopy or MRI). In further embodiments, the platelets can be labeled with a nanoparticle, fluorophore, quantum dot, microcrystal, radiolabel, dye, or gold biolabel. The prevention or reduction of thrombus formation also can be readily determined by methods known to one skilled in the art, which include but are not limited to aggregometry, review of real-time video of blood flow in the animal, and determination of vessel occlusion, as well as by the examples provided below.

[0182] Additionally, the transgenic mouse's bleeding phenotype can be exploited to screen potential prothrombotic compounds, in addition to anti-thrombotics discussed above. A test compound (such as an alleged thrombotic that would induce and/or stimulate blood clotting) can be administered to the animal perfused with human platelets subsequent to vessel injury in order to determine whether blood clotting occurs. In some embodiments, the induction or stimulation of thrombus formation at site of injury upon administration of a compound can be visually examined via tracking the localization of labeled platelets (such as with high resolution in vivo microscopy or MRI). In further embodiments, the platelets can be labeled with a nanoparticle, fluorophore, quantum dot, microcrystal, radiolabel, dye, or gold biolabel. The prevention or reduction of thrombus formation also can be readily determined by methods known to one skilled in the art, which include but are not limited to aggregometry, ex-vivo flow chamber studies, review of real-time video of blood flow in the animal, and determination of vessel occlusion.

[0183] One of ordinary skill in the art can assess that the VWF-A1 mutants of this invention have the required properties of competitive binding to the GPIb platelet receptor in a manner that competes with the native VWF. Suitable assays are set forth in detail in the examples below, including ristocetin-induced platelet aggregation, platelet aggregation induced by ADP, thrombin, collagen, and platelet adhesion in a flow model.

[0184] In one embodiment of the invention, the non-human transgenic animal that expresses a modified A1 domain of the VWF sequence (for example, an amino acid residue substitution at a position involved with binding to GPIb alpha, such as, but not limited to, positions 1263, 1269, 1274, 1287, 1302, 1308, 1313, 1314, 1326, 1329, 1330, 1333, 1344, 1347, 1350, 1370, 1379, 1381, 1385 1391, 1394, 1397, 1421, 1439, 1442, 1449, 1466, 1469, 1472, 1473, 1475, 1479) can be used to validate new devices aimed at determining the effectiveness of antithrombotics in humans.

[0185] The non-human transgenic animal may also be used for determining the effectiveness of gene therapy (for example, assessing whether VWF-A1 protein targeting and protein expression was successful). Gene therapy refers to the insertion of genes into an individual's cells and tissues to treat a disease. For example, in a hereditary disease, a defective mutant allele is replaced with a functional one. The efficiency of VWF-A1 gene transfer by nonviral methodologies (i.e. lipofection) or viral methodologies (such as adenovirus infection described in U.S. Pat. No. 6,927,278 or United States Application publication No. 2005/0169899) can be assessed using the non-transgenic mouse model described above via examining whether replacement of a portion or the whole VWF gene in a subject (such as a mutant VWF mouse of the invention) affects clot formation in vivo. Results obtained from such a mouse model can then be correlated with the likely effect to be observed in human subjects. For gene therapy reviews, see Zuckerbraun et al., (2002) *Arch Surg.* 137(7):854-61; Melo et al., (2004) *Arterioscler Thromb Vasc Biol.* 24(10): 1761-74; and Dulak et al., (2006) *Cell Biochem Biophys.* 44(1):31-42, which are incorporated by reference in their entirety.

[0186] The invention provides a method for testing the efficiency of gene therapy in regulating thrombus formation

in a subject. It also provides a method to test gene therapies directed at correcting genetic mutations associated with von Willebrand disease. The method can comprise the following steps: introducing a vector into the non-human transgenic animal of the invention described above, wherein the vector comprises a nucleic acid encoding a platelet receptor polypeptide, a platelet ligand polypeptide, or a VWF polypeptide, or a portion thereof; allowing sufficient time for expression of the polypeptide; perfusing platelets into the non-human transgenic animal that has one or more mutations in the VWF-A1 domain as previously described under a condition suitable for GPIb-alpha-VWF-A1 protein binding; and identifying an occurrence of a thrombotic event in the animal. For example, the vector introduced into the subject can be an adenovirus or DNA vector described in earlier sections utilizing methods discussed previously (see also Zuckerbraun et al., (2002) *Arch Surg.* 137(7):854-61; Melo et al., (2004) *Arterioscler Thromb Vasc Biol.* 24(10): 1761-74; and Dulak et al., (2006) *Cell Biochem Biophys.* 44(1):31-42). For example, the non-human animal that has one or more mutations in the VWF-A1 domain can be the murine model homozygous for the VWF-A1$^{1326R>H}$ mutation.

[0187] In one embodiment of the invention, the platelets can be human platelets. In particular, the platelets are not murine platelets. In some embodiments, the thrombotic event comprises blood clotting, abnormal bleeding, abnormal clotting, death, or a combination thereof. Such an event can be identified using dynamic force microscopy, a coagulation factor assay, a platelet adhesion assay, thrombus imaging, a bleeding time assay, aggregometry, review of real-time video of blood flow, a Doppler ultrasound vessel occlusion assay, or a combination of the techniques previously described. In a further embodiment, perfusing platelets can be followed by a perfusion of a labeled agent. Non-limiting examples of a labeled agent comprises one or more of a nanoparticle, a fluorophore, a quantum dot, a micro crystal, a radiolabel, a dye, a gold biolabel, an antibody, or a small molecule ligand. In some embodiments, the agent targets a platelet receptor, a VWF protein, or a portion thereof.

[0188] The invention also provides a method to correlate results obtained with an in vitro assay designed to measure the effects of antithrombotics or biomarkers of platelet activation in patients. For example, a biomarker is an indicator of a particular disease state or a particular state of an organism, such as when the subject experiences vascular vessel wall injury. Upon injury to the vessel wall and subsequent damage to the endothelial lining, exposure of the subendothelial matrix to blood flow results in deposition of platelets at the site of injury via binding to the collagen with the surface collagen-specific glycoprotein Ia/IIa receptor. This adhesion is strengthened further by the large multimeric circulating protein VWF, which forms links between the platelet glycoprotein Ib/IX/V and collagen fibrils. The platelets are then activated and release the contents of their granules into the plasma, in turn activating other platelets. For example, Glycoprotein VI (GP6) is a 58-kD platelet membrane glycoprotein that plays a crucial role in the collagen-induced activation and aggregation of platelets. The shedding of GP6 can act as a marker representing that a person is at risk of myocardial infarction. In one embodiment, platelets obtained from a subject determined to have an elevated biomarker level (for example, GP6) can be infused into the non-human transgenic animal described above according to previously described methods, wherein the occurrence of a thrombotic event can be evaluated. In another embodiment, platelets obtained from a subject undergoing an anti-thrombotic treatment can be infused into the non-human transgenic animal described above according to previously described methods, wherein the occurrence of a thrombotic event can then be evaluated.

[0189] Method of Screening and Treating Subjects with Abnormalities of Platelet Function

[0190] The invention provides methods for treating subject with platelet function abnormalities, such as Von Willebrand disease (VWD), Bernard-Soulier syndrome, May-Hegglin anomaly, Chediak Higashi syndrome, and the like. In addition, the invention also provides methods for detecting abnormal platelet function or morphology in a subject.

[0191] VWD is a common hereditary coagulation abnormality that arises from a quantitative or qualitative deficiency of VWF). VWD affects humans, in addition to dogs and cats. There are three types of VWD: type 1, type 2, and type 3. Type 1 VWD is a quantitative defect, wherein decreased levels of VWF are detected but subjects may not have clearly impaired clotting, Type 2 VWD is a qualitative defect, wherein subjects have normal VWF levels but VWF multimers are structurally abnormal, or subgroups of large or small multimers are absent. Four subtypes exist: Type 2A, Type 2B, Type 2M, and Type 2N. Type 3 is rare and the most severe form of VWD (homozygous for the defective gene). (Braunwald et al., *Harrison's Principle of Internal Medicine,* 15 th ed., (Chapter 116) 2001, McGraw Hill, Columbus, Ohio).

[0192] Bernard-Soulier Syndrome is a rare disorder caused by a deficiency of the surface platelet receptor GPIb alpha. As a result, platelets fail to stick and clump together at the site of the injury. Functional abnormalities have also been observed in some hereditary platelet disorders wherein the platelets are of abnormal size or shape, such as in May-Hegglin Anomaly and Chediak Higashi syndrome. (Braunwald et al., *Harrison's Principle of Internal Medicine,* 15th *ed.* (Chapter 116) 2001, McGraw Hill, Columbus, Ohio).

[0193] According to the invention, abnormal platelet function or morphology can be screened in a subject. The method can comprise the following steps: affixing a VWF-A1 molecule to a bottom surface of a flow chamber, or chip (such as a BIAcore chip), wherein the VWF-A1 molecule comprises at least one mutation at a position selected from the group consisting of 1263>S, 1269>D, 1274>R, 1287>R, 1302>D, 1308>R, 1313R>W, 1314>V, 1326>H, 1329>I, 1330>G, 1333>D, 1344>A, 1347>V, 1350>A, 1370>S, 1379>R, 1381>A, 1385>M 1391>Q, 1394>S, 1397>F, 1421>N, 1439>V, 1442>S, 1449>Q, 1466>P, 1469>L, 1472>H, 1473>M, 1475>Q, 1479>G, and any combination thereof, where the position corresponds to an amino acid position of human von Willebrand Factor A1 protein shown in SEQ ID NO: 6; perfusing a volume of whole blood or plasma over the surface-immobilized VWF-A1 molecule complexed to the murine mutant VWF-A1 protein in the flow chamber at a shear flow rate of at least 100 s^{-1}; perfusing a targeted molecular imaging agent into the flow chamber at a shear flow rate of at least 100 s^{-1}; determining whether platelets bind to the surface-immobilized-mutant-murine-VWF-A1 using a diagnostic assay; and comparing diagnostic assay results to a standard control, wherein the standard control sample was subjected to the steps described above. For example, the VWF molecule can be an antibody, a peptide, or a Fab fragment directed to a VWF polypeptide or a portion thereof. In one

embodiment, the molecule can comprise a native or mutant VWF-A1 molecule, a purified native VWF or a mutant plasma VWF.

[0194] In one embodiment, whole blood or plasma sample can be perfused into the chamber or onto the chip, wherein the sample is obtained from the subject. For example, approximately 50 μl of whole blood can be perfused according to the method, or about 100 μl to about 150 μl of plasma can be perfused. As a standard control, the steps of the method described above can be performed using lyophilized non-self platelets, and can be subsequently compared to results obtained using the subjects' platelets. Here, the subject can be a human, a canine, a feline, a murine, a porcine, an equine, or a bovine.

[0195] In one embodiment, the targeted molecular imaging agent can comprise a nanoparticle, a fluorophore, a quantum dot, a microcrystal, a radiolabel, a dye, a gold biolabel, an antibody, a peptide, a small molecule ligand, or a combination thereof. In another embodiment, the targeted molecular imaging agent can bind to a platelet receptor, a platelet ligand, or any region of a VWF protein or a portion thereof. In a further embodiment, the targeted molecular imaging agent can comprise horseradish peroxidase (HRP) coupled to an antibody directed at VWF-A1. Following binding to VWF-A1, a reaction with diaminobenzidine (DAB) can be performed where DAB is reduced by HRP to produce a brown precipitate at the site of binding. This technique allows for enzymatic, calorimetric detection of binding that can be visualized by transmitted light microscopy. For example, if the antibody is directed at a platelet receptor, and calorimetric detection represents whether the antibody bound to the platelet-VWF-A1 complex, the absence of color would denote the lack of a complex formation, thus suggesting that platelets were unable to bind to VWF-A1. The lack of platelet binding could suggest functional defects in the subject's platelets. In one embodiment, platelets bound to VWF-A1 are less than about 500 cells/mm^2.

[0196] The normal platelet morphology is discoid with some spherical shaping. In one embodiment of the invention, the platelets obtained from the subject and that are subsequently screened are substantially spherical in shape. To further analyze platelet morphology, gross platelet histology can be assessed via light microscopy or electron microscopy. In another embodiment, platelets having an abnormal morphology are greater than about 2 μm in diameter. (Ross M H, *Histology: A text and atlas* 3rd *edition,* Williams and Wilkins, 1995: Chapter 9). Various assays can be used to assess whether platelet function is normal, such as a platelet adhesion assay, fluorescence imaging, a chromogenic indication, microscopy morphology analysis, or those listed in *Harrison's Principle of Internal Medicine,* 15th *ed.* ((Chapter 116) 2001, McGraw Hill, Columbus, Ohio), which are hereby incorporated by reference.

[0197] The invention also provides a method of treating abnormalities in clotting due to a defect in the interaction between GPIb alpha and the A1 domain of VWF as occurs in certain types of von Willebrand Disease (VWD), where the method entails administering to the subject an effective amount of a compound that promotes platelet adhesion in the subject, wherein the compound abbreviates off-rate (k_{off}) and/or enhances the on-rate binding kinetics, and or strengthens the bond between VWF-A1 and GPIb-alpha by at least two-fold. Thus, administration of the compound increases blood coagulation in the subject, for example, subjects diagnosed with VWD. In one embodiment, VWD is Type 1 or Type 2. In another embodiment, the compound is one identified by the screening methods described above. Coagulation can be measured by a coagulation factor assay, an ex-vivo flow chamber assay, a platelet adhesion [see EXAMPLES section] or those assays listed in *Harrison's Principle of Internal Medicine,* 15th *ed.* ((Chapter 116) 2001, McGraw Hill, Columbus, Ohio).

[0198] Therapeutic Formulations

[0199] Therapeutic compounds according to this invention are formulated in pharmaceutical compositions containing the compound and a pharmaceutically acceptable carrier. The pharmaceutical composition may contain other components so long as the other components do not reduce the effectiveness of the compound according to this invention so much that the therapy is negated. Some other components may have independent therapeutic effects. Pharmaceutically acceptable carriers are well known, and one skilled in the pharmaceutical art can easily select carriers suitable for particular routes of administration (see, e.g., *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, Pa., 1985).

[0200] The pharmaceutical compositions containing any of the compounds of this invention may be administered by a topical, oral, rectal, parenteral (such as subcutaneously, intramuscularly, intravenously, intraperitoneally, intrapleurally, intravesicularly or intrathecally), or nasal route, as compelled by the choice of drug and disease. One skilled in the pharmaceutical art can discern the optimal route of administration.

[0201] These compounds may also be applied topically or locally, in liposomes, solutions, gels, ointments, biodegradable microcapsules, or impregnated bandages. Compositions or dosage forms for topical application may include suspensions, dusting powder, solutions, lotions, suppositories, sprays, aerosols, biodegradable polymers, ointments, creams, gels, impregnated bandages and dressings, liposomes, and artificial skin.

[0202] Pharmaceutical carriers utilized by one skilled in the art which make up the foregoing compositions include petrolatum, polyethylene glycol, alginates, carboxymethylcellulose, methylcellulose, agarose, pectins, gelatins, collagen, vegetable oils, phospholipids, stearic acid, stearyl alcohol, polysorbate, mineral oils, polylactate, polyglycolate, polyanhydrides, polyvinylpyrrolidone, and the like.

[0203] Therapy dose and duration will depend on a variety of factors, such as the disease type, patient age, therapeutic index of the drugs, patient weight, and tolerance of toxicity. Initial dose levels will be selected based on their ability to achieve ambient concentrations shown to be effective in in vitro models (for example, a dose level used to determine therapeutic index), in vivo models, and in clinical trials. The skilled clinician using standard pharmacological approaches can determine the dose of a particular drug and duration of therapy for a particular patient in view of the above stated factors. The response to treatment can be monitored by analysis of body fluid or blood levels of the compound and the skilled clinician will adjust the dose and duration of therapy based on the response to treatment revealed by these measurements.

EXAMPLES

[0204] A number of Examples are provided below to facilitate a more complete understanding of the present invention.

However, the scope of the invention is not limited to specific embodiments disclosed in these Examples, which are for purposes of illustration only.

[0205] Biophysical and molecular approaches are essential for understanding the structure:function relationship between a receptor and its ligand. Thus, the ability to study such interactions in an appropriate physiological and/or pathological setting is desirable. To do so, one requires an animal model that is amenable to genetic manipulation and has receptor-ligand interactions that closely resemble those found in humans. Thrombosis models in hamsters and guinea pigs have proven useful in pharmacological studies, but a mouse model would prove to be more beneficial based on the ability to insert or delete genes of interest, accessibility of tissues for study, and cost and ease of handling (49, 50). Regarding GPIb alpha-VWF interactions, two groups have significantly advanced the understanding of the importance of these interactions in mediating thrombosis by generating mice deficient in these proteins (51, 52). Yet, no information regarding the role of the biophysical properties of the GPIb alpha-VWF-A 1 in regulating the processes of thrombosis and hemostasis will be obtained. Thus, the next logical approach is to generate animals with mutations within the VWF-A1 domain that change its kinetic properties in a desired manner and to correlate these biophysical alterations with the ability of these mice to maintain adequate hemostasis and to develop thrombi in response to vascular injury. Such information will be useful in designing therapies that reduce or enhance these processes.

Example 1

VWF Characterization

[0206] VWF Microsphere Studies

[0207] The association and dissociation kinetics of the GPIb alpha-VWF-A1 bond and the impact of fluid shear and particle size on these parameters can be determined by measuring the frequency and duration of transient adhesive events, known as transient tethers, that represent the smallest unit of interaction observable in a parallel-platelet flow chamber.

[0208] Production of recombinant VWF-A1 protein and coating of microspheres. The generation of recombinant VWF-A1 protein (residues 1238 to 1472 of the mature, recombinant VWF) and its subsequent coupling to microspheres is performed as previously described (Doggett, T. A. et al. (2002) *Biophys. J.* 83, 194-205). Proper size, purity, and disulfide bonding of all proteins is assessed by Coomasie-blue staining of SDS-PAGE gels run under reducing and non-reducing conditions. Mass spectrometry is also employed to evaluate size and disulfide bonding pattern.

[0209] The resulting recombinant proteins are bound to polystyrene microspheres (goat anti-mouse IgG (FC); Bangs Lab, Inc., Fishers, Ind.) that were initially coated with a saturating concentration of mouse anti-6-HIS antibody as previously described in our publications. We have found this coating method to be superior to direct covalent coupling of the VWF-A1 to the beads as it prevents significant loss in protein function. Estimation of the amount of VWF-A1 bound to the beads is determined using a monoclonal antibody generated in our lab against the human and murine A1 domains, mAb AMD-1 and mAb AMD-2, respectively, and a calibrated microbead system (Quantum Simply Cellular; Flow Cytometry Standards Corp., San Juan, P.R.) following the manufacturer's instructions.

[0210] Laminar flow assays. In flow assays involving protein-coated microspheres, human or murine platelets purified by gel filtration are incubated with 10 mM sodium azide (NaN_3), 50 ng/ml prostaglandin E_1, and 10 μm indomethacin (Sigma Immunochemicals, St. Louis, Mo.) to reduce the possibility of activation and potential alterations in expression and/or distribution of GPIb alpha on their surface. Platelets are subsequently allowed to settle in stasis on Fab fragments of monoclonal antibodies that recognize either human (i.e., mAb 7E3) or murine (i.e., mAb NAD-1) alpha IIb/β_3 in order to form a reactive substrate. The use of platelets in lieu of recombinant proteins or transfected cells as the immobilized substrate enables evaluation of GPIb alpha in its native form (i.e. correct orientation and proper post-translational modification). Platelet coverage of <10% will be bound in this manner can remain relatively unactivated for up to 30 min as evident by morphology on light microscopic examination (FIG. **7**A) and lack of expression of P-selectin by fluorescence microscopy (FIG. **7**B).

[0211] To reduce the possibility of multiple bond formation that would result in a prolongation in interaction times between the beads and immobilized platelets, the lowest site densities of VWF-A1 capable of supporting these brief interactions is used, a value we found to correspond to ~30 molecules μm^2. At this site density, we have shown that the formation of transient tethers between this receptor-ligand pair has distribution of bond lifetimes that obey first order dissociation kinetics. The duration of these interactions are measured by recording images from a Nikon X60 DIC objective (oil immersion) viewed at a frame rate of 235 fps (Speed Vision Technologies, San Diego, Calif.) and subjected to wall shear stresses (WSS) ranging from 0.5 to 3.0 dyn cm^{-2}. The cellular off-rates are determined by plotting the natural log of the number of VWF-A1 coated microspheres that interacted as a function of time after the initiation of tethering, the slope of the line=$-k_{off}(s^{-1})$ which is the inverse of the bond lifetime. The force acting on the tether bond was calculated from force balance equations as stated above and k_{off} plotted as a function of these forces. An example of the measurement of the duration of a transient tether and estimation of off-rates as a function of WSS is demonstrated below for WT human VWF-A1 (FIG. **8**, A-C). To demonstrate that our method for surface immobilization of platelets does not result in an alteration in the kinetics of the GPIb alpha-VWF-A1 tether bond, resting platelets were first fixed in paraformaldehyde prior to immobilization. As these platelets cannot activate, the kinetics should be reflective of GPIb alpha in the resting state. Indeed, analysis of the k_{off} for this interaction using fixed platelets was identical to that observed for platelets treated with metabolic inhibitors (FIG. **8**D).

[0212] The Structure-Function of Murine VWF-A1

[0213] To determine the structure and function of murine VWF-A1, its adhesive interactions with murine and human GPIb alpha, and whether the kinetics of this interaction mimic those reported in studies of its human counterpart, the domain was initially cloned by PCR from purified mouse genomic DNA. For the purpose of generating a mouse with a genetically modified VWF-A1 domain, a100-kb P1 clone was obtained from screening a 129/Svj DNA genomic library (Genomic Systems, St. Louis, Mo.) by polymerase chain reaction (PCR) using primers directed against a 200 bp region of exon 28. Sequence analysis of flanking regions (10 kb in size) as well as the A1 domain itself was performed and compared to those obtained from a BLAST search to confirm

the fidelity of the clone. The deduced single-letter amino acid sequence of mouse VWF-A1 domain (M VWF) is shown compared to its human counterpart (H VWF) and encompasses amino acids 1260 to 1480 (FIG. **9**). The locations of cysteines forming the loop structure are numbered (1272 and 1458) and differences in residues are highlighted in red. Conversion of the arginine (R) in the mouse A1 domain to histidine (H) as found in its human counterpart (blue χ) has been shown to enable mouse VWF to bind human platelets and simultaneously reduce the binding of mouse platelets. Locations of some, but not all, mutations known to affect human VWF-A1 function are also depicted.

[0214] Although the amino acid sequence homology is ~85%, and preliminary studies suggest that functional differences do exist between human and murine VWF-A1 domains. In a Ristocetin-induced platelet aggregation assays, platelet GPIb alpha binding to wild type human VWF or mouse VWF was analyzed in the absence or presence of ristocetin as described by Inbal, et al. (1993, *Thromb. Haemost.*, 70:1058-1062). In this method, platelet rich plasma (PRP) is placed in a clear cuvette containing a stir bar and inserted into the aggregometer. Platelet aggregation is induced by the addition of ristocetin. In this ristocetin-induced platelet aggregation assay (RIPA), we observe that concentrations of this modulator that are known to cause agglutination of human platelets (~1.0 mg/ml) had no such effect using murine PRP (FIG. **10**B). In fact, only at concentrations of $\geqq$2.5 mg/ml was there any evidence of murine platelet aggregation observed (~30%, FIG. **10**C). In comparison, incubation of murine PRP with thrombin resulted in >90% platelet aggregation (FIG. **10**A).

[0215] To better evaluate the above interactions and to compare functional relationships between human and murine VWF with GPIb alpha, VWF from human and mouse plasma was purified and its ability to mediate platelet adhesion in flow was determined. Multimer gel analysis did not reveal any differences between the two species, especially with regard to high molecular weight components (FIG. **11**A). Moreover, surface-immobilized murine VWF could support adhesion of syngeneic platelets (1×10^8/ml) at a shear rate encountered in the arterial circulation (1600 s^{-1}) as observed for the human plasma protein (FIG. **11**B). In contrast, murine VWF did not support significant interactions with human platelets and vice versa. These results suggest that functional and possibly significant structural differences do exist between the A1 domains of murine and human VWF as primary attachment of platelets at this wall shear rate is dependent on its function. Thus, generation of a recombinant murine VWF-A1 domain is required to fully evaluate similarities and/or differences from its human counterpart.

[0216] Recombinant protein was expressed using a bacterial expression vector under the control of an inducible promoter (pQE9, Qiagen). Insertion of the murine fragment containing the majority of the VWF A1-domain (encoding for amino acids 1233 to 1471) into pQE9 produces an amino-terminal fusion protein containing 10 amino acids (including 6× histidine) contributed by the vector. After induction, inclusion bodies were harvested, washed, and solubilized according to previously published methods (32). The solubilized protein was diluted 40-fold in 50 mM Tris-HCl, 500 mM NaCl, 0.2% Tween 20, pH 7.8 and initially purified over a Ni^{2+}-chelated Sepharose (Pharmacia) column. To increase the yield of functional protein, the material purified from the Ni^{2+} column was absorbed to and eluted from a Heparin-Sepharose column (Amersham Pharmacia Biotech).

[0217] The highly purified protein was dialyzed against 25 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.8. SDS-PAGE analysis revealed a prominent protein band of ~34,000 Da under non-reducing conditions (FIG. **12**). The overall yield of protein obtained using the purification methods described above is ~2 mg/l of bacterial cells.

[0218] The protein was subsequently used in a series of in vitro flow chamber assays to assess function. Washed human or murine platelets (5×10^7/ml) were infused through a parallel plate flow chamber containing glass cover slips coated with either human VWF-A1 or mouse VWF-A1 protein (100 μg/ml final concentration) at a shear rate of 800 s^{-1}. After 5 min of continuous flow, adherent platelets were quantified.

[0219] As shown in FIG. **13**A, mouse VWF-A1 protein supported platelet adhesion as efficiently as its human counterpart under physiological flow conditions. To demonstrate the importance of the single disulfide bond formed by C1272 and C1458, reduced (DTT) and alkylated (iodoacetamide) mouse VWF-A1 was prepared and tested in flow. Reduction and alkylation of the protein abrogated attachment of murine platelets in flow. In addition, the limited ability of the native form of the protein to mediate adhesion of human platelets and lack of interaction between human VWF-A1 and mouse platelets suggests that structural/conformational differences exist between the species. However, this does not preclude the study of GPIb alpha-VWF-A1 interactions in mice as both proteins must share common kinetic attributes as they support rapid attachment and translocation of platelets to a similar degree under physiological flow conditions (FIG. **13**B).

[0220] To demonstrate that the presence of the N-terminus His tag does not appear to affect the function of the recombinant protein, we compared the ability of a tagged vs. a non-tagged M VWF-A1 to support mouse platelet adhesion in flow. In the case of the latter, the murine A1 fragment was inserted into pET-11b (Stratagene) and purified as previously described (53). The purified bacterial non-His tag protein was analyzed by SDS-PAGE (12.5%) and found to migrate in an analogous manner to its tagged counterpart under non-reducing and reducing conditions (FIG. **14**A). In addition, no differences were observed in the number of platelets that adhered to and translocated on either protein (449±53 platelets/mm^2 His-tag vs. 423±17 platelets/mm^2 non-His tag) at a shear rate of 800 s^{-1} (FIG. **14**B).

[0221] Characterization of the M VWF-A1 Domain.

[0222] The A1 domains of human and mouse serve an identical purpose: to mediate primary attachment and translocation of platelets in flow. The crystal structure of the mouse A1 domain was solved using recombinant proteins (Fukuda, K., et al., (2005) *Nat. Struct. Mol. Biol.* 12:152-159). The main chain schematic of this domain, with β-strands (arrows) and helices (coils), is shown in FIG. **15**A. The model was built from residues 1270 to 1463 of the murine VWF-A1 crystal. The two cysteines involved in the disulfide bridge are shown as yellow spheres (involving residues 1272 and 1458). The mouse and human A1 domains appear to overlap very closely, which suggests that only minor structural differences may account for the preferential binding of platelets from mice or man to their respective VWF-A1 proteins (FIG. **15**B). In fact the β-sheets of both species are identical within experimental error (a root mean square difference of 0.33 Å for Cα atoms). Thus, minor differences in residues, but not structure, most probably account for the inability of human platelets to interact with mouse VWF-A1 and vice versa.

[0223] Support for this hypothesis is provided by mutagenesis studies. By analyzing the data obtained from the crystal structure of the murine VWF-A1 domain, we have identified several residues that may participate in interactions with GPIb alpha (FIG. **15**C). Residue 1326 was initially chosen for study and was mutated to the corresponding amino acid at the identical location in its human counterpart (from Arg to His). Subsequently, the ability of murine and human platelets to interact with this mutant protein substrate was evaluated at a wall shear rate of 800 s^{-1}. Incorporation of a histidine for arginine at position 1326 in murine VWF-A1 reduced murine platelet adhesion by ~5-fold and increased translocation velocities of cells by ~7-fold as compared to the WT mouse protein (FIGS. **13** and **16**). Interestingly, human platelet interactions with the mutated murine protein were comparable to that of WT human VWF-A1. Conversely, substitution of Arg for His in the human VWF-A1 protein resulted in an increased ability of murine platelets to attach and translocate in a manner similar to that observed for WT murine VWF-A1. These studies support the hypothesis that from a structural and functional standpoint, mouse and human VWF-A1 are very similar.

[0224] Thus, all that remains is to demonstrate that the kinetics of the interaction between the murine GPIb alpha and murine VWF-A1 are similar to its human counterpart and that mutations in man that cause functional alterations in platelet adhesion with VWF have the identical impact on the biophysical properties of the murine receptor-ligand pair.

[0225] The Kinetics of Murine VWF-A1

[0226] To determine whether the kinetics of the murine GPIb alpha interactions with the murine VWF-A1 domain is similar to that of the human receptor-ligand pair, we measured the dissociation of transient tethering events using VWF-A1 coated beads (7 μm diameter) interacting with surface-immobilized platelets. The use of beads with one uniform size and shape permits determination of the relationship between wall shear stress and the force directly acting on the GPIb alpha-VWF-A1 tether bond (F_b), a parameter difficult to estimate for discoid-shaped objects such as platelets. A coating concentration of VWF-A1 was chosen (5 μg/ml corresponding to 30 molecules/μm^2) that supported tether bond formation at wall shear stresses ranging from 0.5 to 3 dyn cm^{-2}. Estimation of the site density of murine VWF-A1 on beads was performed using a monoclonal antibody generated in our laboratory designated as AMD-2. This antibody was made by immunizing Fischer 344 rats (3-4 months old) with recombinant WT protein. Following several injections of murine VWF-A1, serum was collected and screened by ELISA for anti-VWF-A1 antibodies. Spleens from animals with the highest antibody titers were harvested and splenocytes fused with Sp2/0 mouse myeloma cells (54).

[0227] Supernatants of hybridomas were screened for reactivity to mouse VWF-A1 by ELISA (FIG. **17**). Pre-immune rat serum was used as control. Monoclonal antibodies (Mabs) to murine VWF-A1 not only reacted with WT and mutant proteins (1324G>S) but also recognized native VWF purified from mouse plasma. Antibodies are currently being tested for function blocking capabilities to use in both in vitro and in vivo assays. Antibodies will also be used for epitope mapping.

[0228] Analysis of the distribution of interactions times between human or murine VWF-A1 coated beads and their respective platelet substrates, as measured by high temporal resolution videomicroscopy, indicate that >95% of all transient tether bonds events fit a straight line, the regressed slope of which corresponded to a single k_{off} (FIGS. **18**A-C). Notably, the cellular off-rates of these quantal units of adhesion for the WT human and mouse proteins (FIGS. **18**A and B) were quite similar, but were significantly higher than those observed for the murine VWF-A1 containing the type 2B mutation I1309V (1309I>V) (FIG. **18**C). This is consistent with previous results obtained using the same mutation in the human protein (Table 1).

TABLE 1

SINGLE	H1326R*	G1330E*	R1287M*	Q1391P*	A1350T*	S1370G*	D1333A*

[0229] Based on these preliminary results, it appears that the dissociation kinetics of murine GPIb alpha interactions with murine VWF-A1 are nearly identical to its human counterpart and that type 2B mutations also prolongs the bond lifetime of this interaction as seen in man. A complete biophysical analysis is underway and in order to determine values for the intrinsic k_{off} and susceptibility of the bond to force drive dissociation as performed previously for its human counterpart.

Example 2

VWF-A1 Mutagenesis

[0230] Preliminary results indicate that minor differences may exist between murine and human VWF that would preclude one from studying human platelet behavior in a mouse model of thrombosis. However, our findings that the estimated off-rate values and structure of these domains are similar suggest that one can investigate the role of the biophysical properties of the GPIb alpha-VWF-A1 bond in regulating platelet-VWF interactions in vivo using a mouse model. However, neither a delineation of the binding region for GPIb alpha within the murine VWF-A1 domain nor determination of the impact of mutations on the kinetics of this interaction has been performed to date. Thus, both murine and human A1 crystal structures can be exploited to 1) identify candidate residues involved in the binding site for murine GPIb alpha and to determine their impact on the kinetic properties of this receptor-ligand pair, 2) identify residues that confer species specificity, and 3) ascertain whether insertion of known point mutations that cause type 2M and 2B VWD in man alter the kinetic properties of the murine A1 domain in a similar manner. Critical residues can be classified in terms of their impact on the cellular association and dissociation rate constants. Information obtained from these studies can be used to generate mice with mutant A1 domains in order to establish the degree in alteration in the kinetics of the GPIb alpha-VWF-A1 bond that is necessary to perturb hemostasis.

[0231] Similar critical structural elements exist in murine A1 domain to those identified in its human counter-part that contribute to the biophysical properties of the bond formed with GPIb alpha. Thus, to identify structural elements within

the murine VWF-A1 domain that impact on the kinetics of interaction with GPIb alpha, the hypothesis that only minor structural alterations in this domain are responsible for its reduced ability to support interactions with GPIb alpha receptor on human platelets will be tested.

[0232] Site-Specific Mutagenesis of Murine VWF-A1 Domain

[0233] Site-specific mutagenesis of murine VWF-A1 domain will be performed to define residues that contribute to GPIb alpha binding as well as those in that regulate this interaction. Studies will initially focus on amino acids that differ between human and mouse A1 that lie within the vicinity of the proposed GPIb alpha binding pocket.

[0234] To better define residues within murine VWF-A1 that are critical for binding of GPIb alpha on mouse platelets, mutations into VWF-A1 cDNA using a PCR-based strategy will be introduced and the resulting DNA will be sequenced to confirm the presence of the desired mutation(s). Mutations will be based on the murine A1 crystal structure and amino acid substitutions known to affect human VWF function such as those associated with Type 2M or 2B vWD (Tables 2-4). Several surface exposed residues have been identified within the murine A1 domain likely to participate in GPIb alpha binding. These are non-conserved residues in comparison to the human domain. Thus, we will convert these residues at first singly (then doubly and triply), into the murine VWF-A1 to those found in human VWF-A1*. Residues are chosen based on surface-exposure on the front and upper surfaces of the domain as understood by modeling and crystal structure analysis.

TABLE 2

SINGLE	R1326 H*	E1350G*	M1287R*	P 1391 Q*	T1350A*	G1370S*	A1333D*
DOUBLE			R 1287 M* + Q 1391 P*				
TRIPLE			H 1326 R* + G 1350 E* + A1333D*				

TABLE 3

SINGLE	S1289R*	D1323R*	K1348E*	R1392E*

Residues that perturb but do not abrogate platelet binding in the human VWF-A1 protein (Table 3, FIG. **5**B).

TABLE 4

TYPE 2M	G1324S*	Q1367R*	I1369F*	I1425F*
TYPE 2B	R1306L*	I1309V*	V1316M*	R1341L*

Residues chosen based on their ability to abrogate or enhance interactions between human VWF and GPIb alpha (Table 4).

[0235] Type 2B mutations will also be combined with those that dramatically shorten the bond lifetime to determine (increase k_{off}) if these function-enhancing mutations can restore adhesion to that observed for WT VWF-A1.

[0236] Laminar flow assays will be performed to assess the impact of various mutations on platelet adhesion as well as the degree in alteration in the kinetic properties of the GPIb alpha-VWF-A1 bond.

[0237] Murine platelets will be purified as described above and stored in Tyrode's buffer containing 0.25% BSA, pH 7.4. For studies requiring human platelets, blood will be collected by venopuncture from healthy donors and cells obtained from centrifugation of PRP. All platelets will be used within 2 hours of purification.

[0238] To evaluate the impact of mutations on platelet adhesion, both human and murine platelets are perfused over high concentrations of murine VWF-A1 proteins (100 μg per ml) absorbed to glass cover slips in a parallel plate flow chamber at wall shear rates ranging from 20 to 1600 s^{-1}. An enzyme-linked immunosorbent assay is utilized to ensure that an equivalent amount of recombinant WT or mutant protein is immobilized. The number of platelets that attach per unit area per min and their velocity of forward motion (μm/s), termed translocation, is recorded on Hi-8 videotape using an inverted Nikon microscope with a plan 10× or 40× objective, respectively. In addition, whether the incorporated mutations alter the requirement for a critical level of hydrodynamic flow, termed the shear threshold phenomenon, to support interactions of platelets with the reactive substrate can be determined. It has been previously demonstrated that human attachment to immobilized VWF-A1 requires a minimum of >85 s^{-1} of WSR to initiate and sustain this interaction. This phenomenon has also been well described for selectin-dependent adhesion and is believed to rely on a balance between the number of times a receptor encounters it ligand over a defined period of time and the rate at which a bond can form, parameters affected by shear rate, association rate constant, and receptor-ligand concentrations (59, 60). Once attached, however, the bond lifetime influences the velocity at which the cell will move on the reactive substrate in response to shear-induced force (61). Thus, it is likely that several mutations may perturb platelet accumulation on surface-bound VWF-A1 by altering the level of shear flow required to promote platelet attachment as well as the translocation velocities of these cells. For example, it has been shown that the type 2B mutation, Ile1309Val (1309I>V), promotes greater platelet attachment at low shear rates and reduces their translocation velocities as compared to the WT substrate. Similar results were observed upon incorporation of the identical mutation into murine VWF-A1. Demonstration that GPIb alpha on murine platelets is responsible for mediating interactions with recombinant A1 domains can be confirmed by antibody blocking experiments.

[0239] Determination of Tethering Frequencies, Translocation Velocities, Detachment Profiles, and Dissociation Rate Constants Using VWF-A1 Coated Microspheres.

[0240] To better ascertain the alteration in kinetics associated with the proposed mutations, the tethering frequency, translocation velocities, detachment profile, and off-rates of VWF-A1 coated beads (7 μm diameter) interacting with surface-immobilized platelets can be measured. As stated before, the use of beads with one uniform size and shape will permit determination of the relationship between wall shear stress and the force directly acting on the GPIb alpha-VWF-A1 tether bond (F_b), a parameter difficult to estimate for discoid shaped objects such as platelets. Platelet coverage >90% of the glass surface area is used in determining the

tethering frequency (on rate driven phenomenon), translocation velocities (correlates with off-rate) and resistance to detachment forces (measure of bond strength) of VWF-A1 coated beads in flow. Recently, we have demonstrated that comparison of cellular on-rates and apparent bond strengths between WT and mutant forms of VWF-A1 can be achieved by limiting the concentration of these molecules as to prevent multiple bond formation, a process that can mimic an enhancement in either of these kinetic parameters. By using a similar strategy, we can determine whether the proposed mutations will alter the apparent on-rate of the GPIb alpha-VWF-A1 bond by evaluating the frequency of transient tethering events between microspheres coated with low site densities of VWF-A1 proteins and a platelet substrate. Results are expressed as the percentage of beads (per 10× field) that paused, but did not translocate, on over a range of wall shear rates that support such interactions (20 to 400 s^{-1}). Tethers per minute are divided by the flux of beads near the wall per minute to obtain the frequency of this adhesive interaction. Only one tethering event per bead is counted during the observation period.

[0241] For determining translocation velocities, beads (1×10^6/ml), coated with a saturating concentration of VWF-A1 protein are infused into the parallel-plate flow chamber at 1.0 dyn cm^{-2} and allowed to accumulate for 5 min. Subsequently, the wall shear stress is increased every 10 s to a maximum 36 dyn cm^{-2} and the velocities of the beads determined.

[0242] For detachment assays, beads (1×10^6/ml), coated with the minimum but equal amounts of VWF-A1 required to support translocation, are infused into the parallel-plate flow chamber at 1.0 dyn cm^{-2} and allowed to accumulate for 5 min. Subsequently, the wall shear stress is increased every 10 s to a maximum 36 dyn cm^{-2}. The number of beads remaining bound at the end of each incremental increase in wall shear stress is determined and expressed as the percentage of the total number of beads originally bound. Using this strategy we were able to support our claim that type 2B mutations do not strengthen, and in fact may even weaken the interaction between GPIb alpha and VWF-A1 as suggested by the increase in reactive compliance as compared to the native complex (Table 1). In all studies, video images are recorded using a Hi8 VCR (Sony, Boston, Mass.) and analysis of performed using a PC-based image analysis system (Image Pro Plus).

[0243] For determining the kinetics of dissociation, we measure the duration of transient tethers between murine VWF-A1 coated microspheres and immobilized murine platelets as described herein. MC simulations are run and estimates of k_{off} fit to the Bell model by standard linear regression to obtain the intrinsic off-rate (k^0_{off}) and the reactive compliance σ. Results are compared for all mutations to determine their impact on the these kinetic parameters (i.e.—increase or shorten the bond lifetime (k^0_{off}) and/or increase or decrease the susceptibility of the bond to hydrodynamic forces (σ).

[0244] These experiments complement recent work on identifying residues in human VWF-A1 domain critical for interacting with the GPIb alpha. Moreover, they allow for delineation of its binding site in murine VWF-A1. We believe this to be important, as this work will be essential for elucidating the role of the biophysical properties of this receptor-ligand pair in regulating platelet-VWF interactions in vivo. Furthermore, it will pave the way for the generation of mice with comparable types of human vWD (i.e. type 2B) and may even permit the study of human platelets in a mouse model of thrombosis.

[0245] Although our approach to mapping the GPIb alpha binding site is reasonable based on our previous studies, there is no guarantee that the introduction of mutations will not significantly perturb protein structure and thus function. The ability of murine VWF-A1 specific mAbs to recognize mutant proteins should clarify this matter. Similarly, our proposed gain/loss of function experiments involving swapping of residues between human and mouse VWF-A1 will also prove useful in avoiding this pitfall.

[0246] It is important to know whether the regions flanking the mouse A1 domain are important in mediating interactions with GPIb alpha. To this end, we plan to express full-length mouse VWF by inserting it into a mammalian expression vector and transfecting it into COS-7 cells (62). Mutations found to be critical for binding, will be inserted into the full-length construct. As we are in the process of generating the full-length cDNA, we will initially attempt to generate a recombinant protein containing the A1-A2-A3 domains to use in our studies. This will be accomplished using a baculovirus expression system as demonstrated for GPIb alpha.

[0247] The Relationship Between the Major and Minor Binding Sites for GPIb Alpha.

[0248] The recent results on the structure of GPIb alpha and its complex with VWF-A1 domain has not only confirmed our work as well as others with regard to the major binding site for this platelet receptor, but has shed new light into the mechanism by which type 2B mutations may enhance this critical interaction. As shown in FIG. **6**, the concave face of GPIb alpha embraces the A1 domain in two distinct regions. The C-terminal loop of this receptor binds near the top of the domain (major binding site) and the N-terminal region known as the β-finger, at the bottom face (minor binding site) adjacent to the site where type 2B mutations are clustered. Based on these results that type 2B mutations appear to enhance the on-rate (reduced shear rate needed for formation of transient tethers in flow) and prolong the lifetime (5-6 fold) of the interaction between VWF-A1 and GPIb alpha, it is interesting to speculate whether similar alterations in bond kinetics would be observed with type 2B mutations if one interfered with the primary site. For instance, would inclusion of a type 2B with a type 2M mutation reconstitute adhesion, or is some finite interaction time required in the primary binding pocket for GPIb alpha before the effects of these mutations can be observed? These are important questions as they will guide the development of reagents that can either enhance or reduce the interaction between GPIb alpha and VWF-A1.

[0249] The type 2B mutation 1309I>V was incorporated into recombinant human VWF-A1 containing either the type 2M mutation Gly1324Ser (1324G>S) that completely abolishes adhesion or the function reducing mutation His1326Arg (1326H>R) and determined the ability of these doubly mutated proteins to support human platelet adhesion in flow. In comparison to WT, a ~3-fold increase in wall shear rate is required to promote platelet attachment to human VWF-A1 containing Arg at 1326 (FIG. **19**A). Incorporation of the type 2B mutation, however, appeared to enhance the on-rate of this interaction as manifested by an increase in platelet binding at lower levels of shear flow, but not to levels observed for the type 2B mutation alone.

[0250] To determine whether the 1309 mutation would also prolong the lifetime of the interaction with GPIb alpha, the

distribution of interactions times was analyzed between VWF-A1 coated beads and surface-immobilized platelets at a wall shear stress of 1 dyn cm^{-2}. Remarkably, a 2-fold reduction in k_{off} was noted (from 15.6 to 8.5 s^{-1}) as compared to the single, function-diminishing mutation (FIG. **19**B). This value is similar to that of the native receptor-ligand interaction with a k_{off} of 6.5 s^{-1} under identical flow conditions. By contrast, incorporation of Val for Ile at residue 1309 in an A1 domain containing the type 2M mutation 1324G>S did not reconstitute platelet adhesion. Thus, these results suggest that it is essential for bond formation to occur in the primary GPIb alpha binding site (top face of the A1 domain). Moreover, the region of the A1 domain where type 2B mutations are clustered appears to be critical for stabilizing interactions with GPIb alpha. Similar findings were observed for murine VWF-A1 containing the identical type 2B mutation but with a change in Arg to His at residue 1326. A complete biophysical analysis is underway and in order to determine the full extent of the 1326 mutation on the intrinsic k_{off}, the susceptibility of the bond to force drive dissociation, and whether type 2B mutations can restore these parameters to levels obtained for the native receptor-ligand bond. In addition, it would be interesting to determine in vivo whether the enhancement in binding and increase in bond lifetime imparted by the type 2B mutation would correct any perturbation in hemostasis that may occur as a result of impacting on the function of the primary binding site for GPIb alpha.

Example 3

Genetically Modified VWF-A1 Mice

[0251] Recent kinetic evaluation of mutations associated with type 2B and platelet-type vWD suggests that the intrinsic properties of the GPIb alpha-VWF-A1 tether bond contribute to the regulation of platelet interactions with VWF. This is also supported by our preliminary studies investigating the impact of botrocetin on the biophysical properties of this receptor-ligand pair. Thus, by using the information obtained in Example 2, mutations can be incorporated into the murine A1 domain of the VWF gene that increase or decrease the intrinsic on- and off-rates by varying degrees in order to truly understand the importance of these kinetic parameters in controlling platelet adhesion. Moreover, the role of the minor binding site, where the majority of type 2B mutations have been identified, can be further delineated by combining such mutations with those that significantly shorten the lifetime of interaction between GPIb alpha and VWF-A1. Results indicate that substitution of the murine residue Arg at position 1326 for His at the same location in the human A1 domain results in a diminished on-rate as manifested by the increased requirement for shear flow to promote attachment and a significant increase in k_{off} (shortening of bond lifetime). Subsequent incorporation of the type 2B mutation 1309I>V into this mutant domain significantly reverses the functional defect in adhesion and returns the off-rate closer to that observed for the WT domain. Similar results have been obtained with murine VWF-A1 in which Arg was replaced by His at residue 1326. Thus, introduction of these two mutations separately and then together into the mouse VWF gene will be the initial focus. This single amino acid substitution enables the mouse VWF-A1 domain to bind platelets at levels equivalent to its human counterpart. Thus, introduction of the two mutations separately (1326R>H or 1309I>V) and then together into the mouse VWF gene has been the target of recent studies. In addition, replacing >90% of the entire mouse A1 domain with its human counterpart is also central to current investigations. This model will enable one to test all potential therapies directed against this human domain in a mouse model of thrombosis.

[0252] Generation of mice that incorporate mutations into their A1 domain that significantly shorten the bond lifetime will be present with prolonged bleeding times and will be resistant to thrombus formation, while the additional incorporation of a type 2B mutation will correct these abnormalities by prolonging the tether bond lifetime to that observed for the WT domain. This should allow for sufficient time to form multiple bonds between platelets and VWF deposited at sites of vascular injury. Moreover, mice possessing the 1326R>H mutation should be able to support human platelet adhesion at sites of vascular injury. Thus, it will be demonstrated in vivo that the intrinsic properties of the GPIb alpha-VWF-A1 tether bond are indeed critical for regulating the interactions between platelets and VWF at sites of vascular injury.

[0253] By performing a detailed kinetic analysis of mutant VWF-A1 domains prior to the generation of animals with the identical substitutions in amino acids, we have greatly increased the likelihood of altering the interaction between platelets and VWF in a similar manner. We will be able to study the role of the intrinsic properties of the bonds formed between this receptor-ligand pair under complex hemodynamic conditions (i.e. in vivo). The initial choice of the 1326R>H mutation will be of benefit in two ways. First, it allows us to test our hypothesis that a critical interaction time between platelets and VWF is essential to maintaining adequate hemostasis. Secondly, the ability of this mutation to enhance human platelet adhesion will permit us to study their behavior in a mouse model of thrombosis. This is an intriguing concept as it may pave the way to test the impact of various pharmacological agents on human platelet adhesion at sites of vascular injury. For example, a substitution in which a single mutation in murine VWF-A1 (1260 to 1480) can be made in order to achieve human platelet bonding. In addition, a combination of two or more mutations that further perturb the kinetics of the interaction to achieve human platelet binding can also be made. In some instances, the entire mouse A1 domain in the mouse VWF gene with the human A1 domain found in the human VWF gene can be replaced.

[0254] Generation and Characterization of Mice Expressing a Mutant VWF-A1 Domain

[0255] Mutant mouse. As mentioned previously, a 100-kb P1 clone containing the majority of the VWF gene (Genomic Systems, St. Louis, Mo.) was obtained. Digestion with Bam H1 resulted in a ~5.3 kb fragment containing part of intron 28 (including the splice sites), all of exon 28 and part of intron 29 which was the inserted into the pSP72 vector (Promega, WI). This was subsequently digested with Bam H1 and Eco R5 to yield a 2.9 kb (including exon 28) and a 2.4 kb fragment, designated Arm 1 and 2, respectively, both of which were subcloned back into pSP72 vector. This facilitated site-directed mutagenesis of the A1 domain contained within Arm 1. In addition, the 3' end of Arm 1 was extended 2 kb by PCR. Subsequently, Arms 1 and 2 were inserted into a lox P-targeting vector as shown below (FIG. **20**). The fidelity of three constructs containing either the 1309I>V or 1326R>H substitutions or both mutations was confirmed by sequence analysis.

[0256] R1 embryonic stem cells derived from a 129/Sv X 129/Sv-CP F1 3.5-day blastocysts were electroporated with

25 μg of linearized targeting construct and selected in both G418 (26 μmol/L) and gancyclovir (0.2 μmol/L). Genomic DNA from resistant clones were digested with EcoR1 or KpnI, and analyzed by Southern blot hybridization with probe "a" or "b", respectively, to determine if the construct was appropriately targeted (FIG. **20**B). Targeting both the type 2B (Ile1309Val or 1309I>V) mutation and the Arg1326His (1326R>H) mutant constructs, have been successful. In a second step, embryonic stem cell clones that had undergone homologous recombination were transfected with 25 μg of Cre-recombinase-expressing plasmid and selected for G418. Clones in which the neo-cassette was deleted were identified by PCR and injected into C57BL/6 blastocysts (The Siteman Cancer Center Core Facility, Washington University). Male chimeric mice were bred to C57BL/6 Cre-recombinase (+) females to obtain heterozygous animals. Heterozygous mice lacking the neocassette, but containing the 1326R>H mutation, were interbred to obtain wild-type, heterozygous, and homozygous animals. Animals were identified by both Southern analysis (FIG. **21**) and by PCR of the A1 domain (FIG. **22**; red boxed area denotes the conversion of Arg to His).

[0257] Determination of the multimeric composition of murine VWF. For platelet counts, whole blood will be collected into heparinized tubes and 100 μl volumes will be analyzed on a Hemavet (CBC Tech, Oxford, Conn., USA) Coulter Counter. The multimeric structure of murine VWF will be assayed by using the Pharmacia Phast Gel System (Pharmacia LKB Biotechnology). Briefly, samples diluted in 10 mmol/L Tris/HCl, 1 mmol/L EDTA, 2% SDS, 8 mol/L urea, and 0.05% bromophenol blue, pH 8.0, will be applied to a 1.7% agarose gel (LE, Seakem, FMC Bioproducts) in 0.5 mol/L Tris/HCl, pH 8.8, and 0.1% SDS with a stacking gel consisting of 0.8% agarose (HGT, Seakem) in 0.125 mol/L Tris/HCl, pH 6.8, and 0.1% SDS. After electrophoresis the protein will be transferred to a polyvinylidene fluoride membrane (Immobilon P, Millipore) by diffusion blotting for 1 hour at 60° C. The membrane will be blocked with 5% nonfat dry milk protein solution for 1 hour at room temperature. After washing with PBS/T, pH 7.4, the blot will be incubated with a polyclonal antibody raised in rabbits against murine VWF at a dilution of 1:500, washed, and incubated with a goat anti-rabbit horseradish peroxidase (Sigma) diluted 1:2000 in PBS/T. After three washes with PBS/T, the membrane will be incubated with the substrate solution (25 mg 3,3'-diaminobenzidine tetrahydrochloride (Sigma) in 50 mL PBS with 10 μL 30% H_2O_2). The enzyme reaction will be stopped by washing the membrane with distilled water.

[0258] To demonstrate that conversion of Arg to His in the mouse A1 domain at position 1326 did not alter plasma protein levels of VWF nor its ability to form multimers, we performed an ELISA to detect mouse VWF in plasma obtained from WT and homozygous animals (FIG. **23**). As shown in FIG. **23**, plasma levels of VWF from homozygous mice (KI) were comparable to WT at all dilutions tested. Moreover, multimer gel analysis of plasma VWF revealed an identical banding pattern between mouse and human VWF. Incorporation of His at position 1326 in the mouse A1 domain had no effect on multimerization of VWF (FIG. **24**). Thus, we are the first to successfully introduce a point mutation into mouse VWF A1 domain.

[0259] Bleeding time for human platelet-induced hemostasis: This assay provides an indirect measure of the ability of platelets and VWF to support hemostasis by interacting with the injured vessel wall. It also indirectly determines the function of multiple receptors and ligands on platelets that are required to form a hemostatic plug. That said, it provides direct evidence that the bleeding defect in our animals can be corrected by the administration of human platelets (FIG. **12**). It is performed by immersing the severed tip (10 mm) of the animal's tail in isotonic saline at 37° C. and monitoring the length of time required for bleeding to cease. Homozygous mutant mice will be infused with an equal volume of either saline or purified human platelets. Platelet specific antibodies or drugs will be administered as described above and their ability to prolong bleeding time evaluated. All experiments will be stopped at 10 min by cauterizing the tail (51).

[0260] Although homozygous mice bearing the 1326R>H mutation are viable, they demonstrate a bleeding phenotype similar to that of animals deficient in VWF (KO) only when 10 mm of the tail is cut (FIG. **25** and FIG. **35**B). Over 90% of these mice bled for a minimum of 8 minutes (end point) in contrast to 3.5 minutes for WT animals as measured by severing 1 cm of their tail. Moreover, thrombus formation induced by perfusion of whole blood from mutant mice over surface-immobilized collagen in vitro was reduced by ~80% as compared to WT controls (FIG. **26**). To further characterize the bleeding phenotype depicted in FIG. **35**, standard techniques known in the art were employed, which involves removal of a minimal amount of the animal's tail (~5 mm). A slight, but statistically significant ($P<0.01$), increase in bleeding was observed in the homozygous 1326R>H mutant mice as compared to WT littermates, but not to the extent of the VWF knockout mouse (a mouse model that bleeds profusely due to complete lack of this plasma glycoprotein). In the homozygous 1326R>H mutant mouse, a larger cut (~10 mm) is needed in order to see a bleeding phenotype comparable to the VWF deficient mouse. Since these mice continue to bleed, the experiment is stopped at 8 min in order to prevent death. This phenotype observed in the 1326R>H mutant mouse is similar to the bleeding observed in human patients who have type 2M VWF disease. As not all type 2M mutations result in a complete loss of interaction between GPIb alpha and VWF-A1, but resemble the adhesion defects outlined for the 1326R>H mutant mouse, this genetically modified mouse model will be useful for directing therapies aimed at patients that have a partial but not complete defect in binding between GPIb alpha and VWF-A1.

[0261] Platelet Adhesion Studies in Mice Expressing a Mutant VWF-A1 Domain

[0262] One goal of the work is generate mice with mutant A1 domains that alter the kinetics of its interactions with GPIb alpha on mouse platelets. The first mutation we chose to introduce was the substitution of histidine for arginine at position 1326. This mutation was chosen based on our crystal structure analysis of the mouse and human A1 domains, which suggested that the location of this amino acid is central to GPIb alpha binding. Mice bearing this mutation are viable and demonstrate a bleeding phenotype, albeit not as severe as those lacking VWF (VWF KO) (FIG. **35**). This was not unexpected as VWF is still present, but has a reduced ability to interact with platelets at high shear rates (>1600 s^{-1}). FIG. **27** demonstrates reduced thrombus formation that occurs when whole blood from these knock-in animals is perfused over collagen-coated cover slips at a shear rate of 1600 s^{-1}. Results thus far indicate a 70% reduction in thrombi formed on collagen as compared to WT controls.

[0263] To demonstrate that the 1326R>H mutant in the A1 domain of mouse VWF (wherein the mutant VWF-A1 domain comprises SEQ ID NO: 5) is far superior to promoting interactions with human platelets under physiologic flow conditions, anticoagulated human blood was infused over surface-immobilized WT or mutant mouse plasma VWF at 1600 s^{-1} (FIG. **28**). Results indicate that the mutant form of mouse VWF can support human platelet attachment to levels observed for its human counterpart, thus making it an ideal system to evaluate human platelet behavior and the impact of novel anti-thrombotic drugs in an animal model. Moreover, this observation was not limited to ex vivo studies, as homozygous mutant mice infused with human (FIG. **29**A) but not mouse platelets (FIG. **29**B) were able to generate an arterial thrombus that occludes the vessel lumen in response to laser-induced vascular injury as depicted by intravital microscopy (transmitted light). Preliminary results indicate that mouse and human A1 domains are structurally similar and serve an identical functional role in the initiation of thrombus formation. Moreover, the ability of the "humanized" mouse A1 domain to support human platelet adhesion to the same degree as its human VWF-A1 counterpart ex-vivo, as well as its preferential binding of human platelets in vivo (FIG. **29**A), suggests that our animal model will be an ideal system for preclinical screening of therapies directed at limiting the interactions between GPIb alpha and the VWF-A1 domain. Moreover, as both hemostasis and thrombosis also rely other key adhesion receptors on human platelets, such as those that interact with collagen OR fibrinogen FIG. **1**B), this model can also be used for testing therapies directed against other human platelet receptors and ligands critical for these processes.

[0264] To assess the biological significance of this finding in terms of its effect on hemostasis, homozygous mutant animals received an infusion of blood-banked human platelets and bleeding time was subsequently measured by severing 1 cm of their tail. Average bleeding time for mice that received human platelets was ~3 minutes vs. 10 minutes (end point) for animals given an intravenous infusion of a physiological buffered saline solution (FIG. **30**). Results indicate that not only can the mutant form of mouse VWF support human platelet adhesion both ex-vivo and in vivo assays, but it can also perform its biological function; supporting hemostasis in the context of vascular injury.

[0265] Evaluation of platelet-VWF behavior in flow. Blood will be collected by cardiac puncture from anesthetized mice and thrombin-mediated activation prevented by the addition of hirudin (160 U/ml, Sigma) (68). Platelet adhesion to a glass cover slip coated with 100 μg/ml of equine tendon collagen (Helena Laboratories, Beaumont, Tex.) will be assessed in a parallel-plate flow chamber apparatus. Whole blood will be infused through the chamber at a wall shear rate of 1600 s^{-1} for 3 minutes. As platelet adhesion under these homodynamic conditions requires VWF deposition and subsequent interactions between its A1 domain and GPIb alpha, the extent of platelet coverage should provide a gross estimate of the degree in impairment between this receptor-ligand pair. In addition, plasma VWF will be purified from these animals to evaluate platelet attachment to this immobilized substrate in flow. The surface area covered by adherent platelets at the end of each experiment will be determined (Image Pro Plus software) and expressed as a percentage of platelet coverage using blood from WT littermates. To better isolate GPIb alpha-VWF A1 interactions, identical experiments can be performed using platelets isolated from alphaIIb beta 3 deficient animals and reconstituting them in platelet poor plasma from our mutant A1 knock-in mice.

[0266] Evaluation of platelet-VWF behavior in vivo. In addition to the proposed in vitro work, platelet-VWF interactions in vivo will also be studied using intravital microscopy (Falati et al. (2002) *Nature Medicine* 8(10): 1175-80). This is accomplished by using a murine model of thrombosis that involves laser-induced injury to micro-vessels contained within the mouse cremaster muscle. The surgical preparation of animals, insertion of lines for administration of cells and anesthesia, will be performed as previously described (69). Human platelets will be collected and prepared, fluorescently labeled, perfused into a mouse model (such as the transgenic mouse of the current invention) via an intravenous injection (Pozgajova et al., (2006) *Blood* 108(2):510-4).

[0267] Surgical preparation of animals: Insertion of lines for administration of cells and anesthesia. Briefly, the skin covering the scrotum will be incised and the intact cremaster muscle dissected free from the connections to the subcutis. The mouse will be placed on a custom-built plexiglass board, and the exposed muscle positioned on a heated circular glass coverslip (25 mm) for viewing. The muscle will be slit along the ventral surface (using a thermal cautery), the testis excised, and the muscle spread across the coverslip with attached sutures (6/0 silk) (FIG. **31**). The cremaster muscle will be kept continuously moistened by superfusion throughout the experiment with sterile, bicarbonate-buffered (pH 7.4), saline solution (37° C.) that is pregased with a 5% CO_2, 95% N_2 mixture for O_2 depletion. All parts of the setup in contact with the superfusion buffer will be presoaked with 1% Etoxaclean (Sigma Chemical Co., St. Louis, Mo.) overnight followed by extensive rinsing in 70% ethanol and endotoxin-free distilled water. The number of mice used for these experiments will be kept to the minimum necessary to establish statistically significant observations. Anesthetized animals will be euthanized after each experiment by CO_2 inhalation.

[0268] Vascular trauma will be generated as follows: The segment of an arteriole will be visualized and recorded as "pre-injury". Subsequently, endothelial damage will be induced via a pulsed nitrogen dye laser at 440 nm applied through the microscope objective using the Micropoint laser system (Photonics Instruments, St. Charles, Ill.). The duration of exposure of the endothelium to the laser light will be varied to produce either a mild injury that supports the formation of a platelet monolayer or significant injury resulting in thrombus formation. The region of interest will then be videotaped and analyzed as described below.

[0269] For example, vascular damage can subsequently be induced in arterioles contained within the cremaster muscle of mice by either 1) pulsed nitrogen dye laser applied through the objective of an intravital microscope (FIG. **32**) or 2) standard application of a ferric chloride solution (Furie et al. *J. Clin. Invest.* 2005; 115:3355). The latter method has the advantage of exposing significant more subendothelial collagen, which will be beneficial for testing the role of the collagen receptors α2β1 in thrombus formation.

[0270] For studies analyzing the dynamic interactions between individual platelets and the injured vessel wall (attachment, translocation, and sticking), cells purified from genetically altered mice will be labeled ex-vivo with a derivative of carboxyfluorescein (BCECF, Molecular Probes) (Diacovo et al. *Science.* 1996). A human thrombus generated in the mutant mouse can also be visualized by this technique,

thus allowing to distinguish human platelets from endogenous circulating mouse platelets upon illumination with an appropriate laser light source (see FIG. **40**). Cells (1×10^7/g of BWT) will be subsequently injected intravenously into mice bearing WT mouse (control) or the "humanized" A1 domains and their behavior visualized in the microcirculation using an intravital microscope (Zeiss, Axiotech Vario; IV500, Mikron Instruments, San Diego, Calif.; and the like) equipped with an iXON EM camera or a silicon-intensified camera (VE1000SIT; Dage mti, Michigan City, Ind.), a Yokogawa CSU22confocal head, and a 488 nm laser line (Andor Technology, Revolution series). A Xenon arc stroboscope (Chadwick Helmuth, El Monte, Calif.) will serve as the light source and fluorescent cells will be viewed through 60× or 100× water immersion objectives (Acroplan, Carl Zeiss Inc.). A tethered platelet will be defined as a cell establishing initial contact with the vessel wall (FIG. **33**A, panel 2-3; FIG. **33**B). The translocating fraction will be defined as number of tethered platelets that move at a velocity significantly lower than the centerline velocity for >1 s. The sticking fraction will be defined as the number of translocating cells that become stationary for >30 s post-tethering. Second order arterioles (up to 50 μm in diameter) will be evaluated for platelet interactions before and after the injury. Evaluation of platelet circulation in larger arterioles may be less accurate secondary to hemoglobin-mediated quenching of fluorescence emitted from platelets traveling in an area of the blood stream distal to the focal plane of the objective. Epi-illumination will only be used during video recordings to minimize possible phototoxic effects on tissue.

[0271] A role for GPIb alpha as well as the collagen ($\alpha2\beta1$) and the fibrinogen (αIIb $\beta3$) receptors can be evaluated by using function-blocking antibodies to these proteins. Moreover, FDA approved anti-thrombotics (such as clopidogrel and tirofiban) can be examined as to whether the drugs inhibit human platelets from forming a thrombus in vivo, validating the mouse model for use in pre-clinical screening. The effect that antibodies and drugs have on altering the interaction between GPIb alpha-VWF-A1 interaction is determined by evaluating whether thrombus formation in the proposed mice is reduced or augmented upon arteriolar injury (FIG. **34**).

[0272] For all experiments, the centerline erythrocyte velocity (Vrbc) is measured using an optical doppler velocimeter (Microcirculation Research Institute, Texas A&M College of Medicine, College Station, Tex.) prior to and after inducing the injury. Shear rate (SR) is then calculated based on Poiseulle's law for a Newtonian fluid: SR=8(Vmean/Dv), where Dv is the diameter of the vessel and Vmean is estimated from the measured Vrbc (Vmean=Vrbc/1.6).

[0273] Characterization of thrombus formation: Thrombus formation can be characterized as follows: (1) Early individual platelet interactions with the damaged vessel wall (number of fluorescently labeled human platelets that attach during the first minute post-injury); (2) time required for thrombus generation of >20 μm diameter; (3) the ability of thrombi to remain at the initial site of vascular injury and not break free (measure of stability); (4) time until vessel occlusion; and (5) site of vessel occlusion, that is, at the site of injury or downstream from it. Platelet-vessel wall interactions can be viewed through 40× or 60× water immersion objectives. To standardize in vivo conditions, the velocity of flowing blood (shear rate) pre-injury is determined by measuring the centerline erythrocyte velocity (Vrbc) using an optical doppler velocimeter. Shear rate (SR) can then be calculated based on Poiseulle's law for a Newtonian fluid: SR=8×(Vmean/Dv), where Dv is the diameter of the vessel and Vmean is estimated from the measured Vrbc (Vmean=Vrbc/1.6). Vessel and thrombus diameters are measured using imaging software (ImagePro Plus).

[0274] Administration of antibodies: Function-blocking monoclonal antibodies 6D1 (anti-human GPIb alpha), 6F1 (anti-human $\alpha2\beta1$) and 7E3 (anti-human αIIb$\beta3$) have been generously provided by Dr. Barry Coller (Rockefeller University, NY). All antibodies are converted to F(ab')2 fragments to limit Fc receptor interactions in vivo. An intravenous dose of 10 μg/g body weight is given approximately 10 minutes after the injection of human platelets but 30 minutes prior to inducing vascular injury. Non-function blocking antibodies to these receptors is used as negative controls and administered under identical conditions. To ensure optimal ligand availability for the collagen and fibrinogen receptors on human platelet, mice possessing the A1 domain mutation have been bred with animals genetically deficient in $\alpha2\beta1$ or αIIb$\beta3$. Thus, endogenous platelets in these animals not only have a reduced ability to interact with the VWF-A1 domain, but also are incapable of binding to collagen or fibrinogen, respectively. Although human platelets have been shown to circulate in mice for a maximum of 24 hours, we can ensure that an equivalent percentage of human platelets are present at the time of vascular injury under each experimental condition (Xu et al. *J. Clin. Invest.* 2006; 116: 769). Thus, 50 μl is obtained from an inserted venous catheter and flow cytometric analysis will be performed to determine the percentage of circulating fluorescently-labeled human platelets.

[0275] Administration of drugs: In comparison to aspirin, clopidogrel (Plavix) is the second most commonly used antithrombotic drug that targets one of the ADP receptors (P2Y12) on platelets, causing irreversible inhibition (Hankey et al. *Med. J. Aust.* 2003; 178:568). ADP is a potent mediator of platelet activation and aggregate formation, and thus considerable effort and funds have been devoted to inhibiting this activation pathway in platelets. Clopidogrel was approved by the FDA in 1997 for clinical use and was found to be of benefit in the secondary prevention of major vascular events in patients with a history of cerebrovascular and coronary artery diseases and major cardiac events post coronary artery stent placement (Gachet et al. *Semin. Thromb. Hemost.* 2005; 31:162). Disadvantages of this drug are: 1) It must be metabolized in the liver to generate an active metabolite, thus limiting its effectiveness in acute settings, and 2) irreversible inhibition that results in a marked prolongation of bleeding time.

[0276] Clopidogrel has been shown to reduce thrombus size and delay its formation in mice with a maximal effective dose of 50 mg/kg given the day before and 2 hours prior to experimentation (Lenain et al. *J. Throm. Haemost.* 2003; 1:1133). This drug will be obtained from the hospital pharmacy and tablets will be dissolved in sterile water for oral administration. Control animals will receive water in lieu of drug. The effectiveness of this treatment regime will be confirmed by first measuring the responsiveness of platelets isolated from drug-treated WT animals to ADP-induced aggregation using an optical aggregometer (Chrono-Log Corp.) as previously described (Leon et al. *J. Clin. Invest.* 1999; 104: 1731). As our mutant VWF-A1 domain mice also have a defect in platelet aggregation, these animals cannot be used for the purpose of testing to ADP-induced aggregation ex-vivo. However, this additional phenotype will be advantageous for us as it limits potential competition between human

and mouse platelets for binding to ligands exposed at sites of vascular injury. Human platelets will be administered 30 minutes prior to vascular injury and 50 μl of blood drawn to determine the percentage of circulating cells as described above. Platelet rich plasma will also be purified from control and drug treated animals that receive human platelets to evaluate the effectiveness of clopidogrel on preventing ADP-induced aggregation of these cells ex-vivo.

[0277] Tirofiban (Aggrastat) is a non-peptide inhibitor of the fibrinogen receptor αIIbβ3 that limits the ability of platelets to form aggregates, an event required for thrombus progression. It has a plasma half-life of approximately 2 hours but only remains bound to platelets for seconds, thus necessitating continuous intravenous administration. It is currently approved for short-term treatment of patients with acute coronary syndrome that require interventional catheterization. Thus, the animals will be dosed based on that given for interventional procedures such as angioplasty, which consists of a 25 μg/kg bolus over 3 minutes followed by a continuous maintenance infusion of 0.15 μg/kg/min until the completion of the experiment (Valgimigli et al. *JAMA*. 2005; 293:2109). Human platelets will be administered 30 minutes prior to vascular injury and 50 μl of blood drawn to determine the percentage of circulating cells as described above.

[0278] Platelet donors. Mice are used as platelet donors. A means to evaluate murine platelet interactions with wild type and mutant VWF-A1 proteins is via in vitro flow chamber assays. Blood from ~10 mice are required to purify adequate numbers of platelets per assay. Blood from donor animals is obtained from the retro-orbital plexus using a heparinized glass pipette. Mice will be anesthetized with Ketamine and Xylazine prior to the procedure and are euthanized by CO_2 inhalation upon completion.

[0279] Bleeding time for human platelet induced hemostasis. This assay is carried out as described above.

[0280] Solution-phase binding assay. For type 2B mutant VWF, its capacity to bind to platelet GPIb alpha in solution can be determined. Plasma is harvested from these mice and VWF purified. Various concentrations of the plasma glycoprotein will be indirectly labeled using a non-function blocking, ^{125}I-labeled mAb to its A1 domain as previously described (67). After a 30 min. incubation, a quantity of this mixture will be incubated with platelets purified from beta 3 deficient mice so to prevent integrin-mediated binding to VWF. After incubation period of 1 hour, an aliquot of this mixture will be added to a sucrose gradient and centrifuged to pellet the platelets. Radioactivity associated with the pellet vs. supernatant will be determined in a γ-scintillation counter, and the binding estimated as the percent of total radioactivity.

Example 4

Defining the In Vivo Role of the von Willebrand Factor A1 Domain by Modifying a Species-Divergent Bond

[0281] Proteins containing von Willebrand Factor (VWF) A domains contribute to human health and disease by promoting adhesive interactions between cells (Whittaker, C. A., & Hynes, R O. *Mol. Biol. Cell.* 13, 3369-3387 (2002)). The VWF-A1 domain is thought to play a critical role in hemostasis by initiating the rapid deposition of platelets at sites of vascular damage by binding to the platelet receptor glycoprotein Ib alpha (GPIbα) at high shear rates (Roth, G. J. *Blood* 77, 5-19 (1991); Cruz, M. A., et al., *J. Biol. Chem.* 268, 21238-21245 (1993); Sugimoto, M. et al., *Biochemistry* 30, 5202-5209 (1991); Pietu, G. et al., *Biochem. Biophys. Res. Commun.* 164, 1339-1347 (1989)). Although congenital absence of VWF in humans has established a role for this plasma glycoprotein in hemostasis (Sadler, J. E. et al. *J. Thromb. Haemost.* 4, 2103-2114 (2006)), the contribution of its A1 domain in clot formation has been questioned in a mouse model of vascular injury (Denis, C. et al. *Proc. Natl. Acad. Sci. USA* 95, 9524-9529 (1998)).

[0282] In this example, murine plasma VWF or its A1 domain fails to support significant interactions with human platelets (and likewise human VWF with murine platelets) under flow conditions. Atomic models of GPIbα-VWF-A1 complexes suggest that the structural basis for this behavior arises primarily from an electrostatic "hot-spot" at the binding interface. Introduction of a single point mutation within this region of murine VWF-A1 is sufficient to switch its binding specificity from murine to human platelets. In addition, introduction of a single point mutation within the electrostatic "hot-spot" region of human VWF-A1 is sufficient to switch its binding specificity from human to murine platelets. Moreover, mice possessing the 1326R>H mutation in their VWF have a bleeding phenotype distinct from VWF-deficient animals, and can be corrected by the administration of human platelets. Mechanistically, mutant animals can generate but not maintain thrombi at sites of vascular injury, whereas those infused with human platelets can form stable thrombi, a process that relies on GPIbα-VWF-A1 interaction. Thus, interspecies differences at protein interfaces can provide insight into the biological importance of a receptor-ligand bond, and aid in the development of an animal model to study human platelet behavior and drug therapies.

[0283] Methods

[0284] Generation of $VWF^{1326R>H}$ mice. The $VWF^{1326R>H}$ targeting vector (FIG. **38**A) was prepared from a 129/SvJ mouse genomic library. The clone was identified by PCR using primers specific for exon 28 of the mouse VWF gene and sequence fidelity of the region to be targeted validated by comparison to published sequence for chromosome 6 (GenBank accession number NW_001030811). The targeting vector is identical to the corresponding region in the mouse genome, except the 1326R>H mutation was created in exon 28 and the Neo cassette flanked by loxP sites was inserted into intron 28. This resulted in the loss of an EcoRV site and the introduction of a new EcoR1 and two new XhoI sites. The construct was electroporated into an embryonic stem (ES) cell line, and potential clones identified by continued growth of cells in G418 and Gancyclovir supplemented media. DNA was isolated from surviving colonies, digested with EcoRI, and screened by Southern analysis using a 1.5 kb probe (A) corresponding to a DNA sequence downstream of the targeting construct. Chimeric mice generated from $VWF^{1326R>H}$ targeted ES cell lines were subsequently bred to a Cre transgenic mouse (C57BL/6 background) and animals containing the 1326R>H mutation, but without the Neo cassette, subsequently identified by both PCR and Southern analysis. WT and homozygous animals were the product of matings between heterozygous mice.

[0285] Analysis of VWF transcripts, antigen levels, multimers, and collagen binding. Detection of transcripts from the A1-A2-A3 domains of murine VWF was performed by RT-PCR. Briefly, mRNA was isolated from lung tissue harvested from either homozygous $VWF\text{-}A1^{1326R>H}$ mice or aged-mated WT littermate controls (Oligotex®, Qiagen). Genera-

tion of cDNA and PCR-amplification of desired transcripts was performed using SuperScript™ One-Step RT-PCR (Invitrogen) and oligos specific for the A domains of VWF.

[0286] Functional factor VIII levels were determined by a mechanical clot detection method using the STA automated coagulation analyzer (Diagnostica Stago, Parsippany, N.J.). A log-log calibration curve was established by measuring the activated partial Thromboplastin time (aPTT) of varying dilutions of reference plasma. The aPTT of a 1:10 dilution of sample plasma mixed with factor VIII deficient plasma was determined, compared to the calibration curve, and the activity expressed as a percent of normal.

[0287] Evaluation of VWF antigen levels was performed as previously described (Denis, C. et al. *Proc. Natl. Acad. Sci. USA* 95, 9524-9529 (1998)). For multimer analysis, plasma from sodium citrate treated whole blood was diluted 1:5 in electrophoresis sample buffer (final concentration 10 mM Tris-HCl pH 8.0, 2% SDS, 1 mM EDTA) and heated at 56° C. for 30 minutes. Electrophoresis was carried out overnight (64 volts, 15° C.) through a horizontal SDS-agarose gel in 1.2% agarose (Ruggeri, Z. M. & Zimmerman, T. S. *Blood* 57, 1140-1143 (1981)). The gel was then electrophoretically transferred (150 mA, 90 minutes) to Immobilon (Millipore) followed by blocking (2 h) with 5% powdered milk in TBST (Tris HCl pH 8.0, 0.15M NaCl, 0.05% Tween-20). The membrane was incubated with a 1:500 dilution of rabbit anti-human VWF antiserum (DAKO) for 1 h, washed in TBST, and then incubated with a 1:10,000 dilution of HRP-conjugated mouse anti-rabbit IgG (Calbiochem). Bands were subsequently detected by chemiluminescence system (GE Healthcare). For comparison, a sample containing pooled human plasma from normals or patients with type 2B VWD was also loaded on the gel. Binding of VWF to surface-immobilized collagen was performed as previously described (Smith, C. et al. (2000) *J. Biol. Chem.* 275, 4205-4209). Briefly, 100 μg/ml of acid soluble type I collagen from human placenta (Sigma) was added to a 96 well microtiter plate and allowed to incubate overnight (4° C.). After washing and blocking with TBS containing 3% BSA and 0.05% Tween 20, varying concentration of platelet poor plasma harvested and pooled from WT, homozygous $VWF^{1326R>H}$, and VWF deficient mice was added to the wells and incubated for 1 h (37° C.). Wells were then washed and bound VWF detected by an ELISA as described above.

[0288] Ex vivo platelet adhesion studies. Experiments were performed in a parallel-plate flow chamber as previously described (Offermanns, S. (2006) *Circ. Res.* 99, 1293-1304). For studies involving plasma VWF, a polyclonal anti-VWF antibody (Dako) was absorbed overnight (4° C.) to a six well tissue culture plate. Subsequently, the plate was washed and non-specific interactions blocked by the addition of TBS containing 3% BSA, pH 7.4 (1 h, 37° C.). Human or murine plasma obtained from heparinized whole blood was added and the plates placed at 37° C. for an additional 2 h. Generation, purification, and surface-immobilization of recombinant VWF-A1 proteins was performed as previously described (Doggett, T. A. et al. *Biophys. J.* 83, 194-205 (2002)). Both human and murine VWF-A1 constructs consist of amino acid residues 1238 to 1471, with a single intra-disulfide bond formed between residues 1272 and 1458 and were generated in bacteria. Citrated whole blood (150 μl) collected via cardiac puncture from anesthetized homozygous $VWF^{1326R>H}$ or WT mice or from venopuncture from human volunteers was perfused over the immobilized substrates at a wall shear rate of 1600 s^{-1} for 4 min, followed by washing with Tyrode's buffer under the identical flow conditions. The number of platelets attached per unit area (0.07 mm^2) and translocation velocities were determined by off-line analysis (Image-Pro Plus, Media Cybernetics). For GPIbα inhibition studies, the function-blocking mAb 6D1 (20 μg/ml) or mAb SZ2 (20 μg/ml; Beckman Coulter) was added to anticoagulated human blood for 30 min prior to use. Experiments were performed in triplicate on two separate days. An ELISA was used to ensure equivalent coating concentration of plasma and recombinant proteins (Denis, C. et al. (1998) *Proc. Natl. Acad. Sci. USA* 95, 9524-9529).

[0289] In vivo thrombus formation. Administration of anesthesia, insertion of venous and arterial catheters, fluorescent labeling and administration of human platelets (5×10^8/ml), and surgical preparation of the cremaster muscle in mice have been previously described (Doggett, T. A. et al. *Biophys. J.* 83, 194-205 (2002); Diacovo, T. G., et al., *Science* 273, 252-255 (1996)). Injury to the vessel wall of arterioles (~40-65 μm diameter) was performed using a pulsed nitrogen dye laser (440 nm, Photonic Instruments) applied through a 20× water-immersion Olympus objective (LUMPlanFl, 0.5 NA) of a Zeiss Axiotech vario microscope. Mouse platelet- and human platelet-vessel wall interactions were visualized using either bright field or fluorescence microscopy. The latter utilized a fluorescent microscope system equipped with a Yokogawa CSU-22 spinning disk confocal scanner and 488 nm laser line (Revolution XD, Andor™ Technology). The extent of thrombus formation was assessed for 2 min post injury and the area (μm^2) of coverage determined (Image IQ, Andor™ Technology). For GPIbα or αIIbβ3 inhibition studies, the function-blocking mAb 6D1 or 7E3 (20 μg/ml), respectively (from B. Coller, Rockefeller University), was added to purified human platelets for 30 min prior to administration.

[0290] Tail bleeding assay. Bleeding times were measured in 7-week old mice after amputating 1 cm of the tail tip as previously described (Denis, C. et al. (1998) *Proc. Natl. Acad. Sci. USA* 95, 9524-9529). In studies involving human platelets, platelet concentrates were obtained from Columbia Presbyterian Hospital Blood Bank, washed and resuspended in normal saline (1.5×10^9/300 μl) before administering through a catheter inserted into the right internal jugular vein. Tail cuts were performed 5 min after completion of the infusion of platelets. PLAVIX and ReoPro® were obtained from the research pharmacy at CUMC. For studies involving PLAVIX, animals received a 50 mg/kg oral dose of the drug the day before and 2 h prior to the administration of human platelets. ReoPro® was given initially as an intravenous bolus (0.25 mg/kg) 5 min after the administration of human platelets, followed by a continuous infusion (0.125 μg/kg/min) as per the manufacturer's recommendations.

[0291] Structural Modeling. There are three crystal structures of the GPIbα-VWF-A1 complex: two are WT except for mutated N-glycosylation sites in GPIbα (Fukuda, K. et al., (2005) *Nat. Struct. Mol. Biol.* 12, 152-159; Dumas, J. J. et al. (2004) *J. Biol. Chem.* 279, 23327-23334), and one is a gain-of-function mutant (Huizinga, E. G. et al. (2002) *Science* 297, 1176-1179). The structures have only small differences that are not the result of the presence of mutations or botrocetin binding (Fukuda, K., et al., (2005) *Nat. Struct. Mol. Biol.* 12, 152-159). Both N-glycosylation sites in human GPIbα lie on the well-ordered upper ridge of the LRR, 18 Å and 27 Å (Cα-Cα) from the nearest VWF-A1 residue, so their absence

is unlikely to affect the structure of the complex. Murine GPIbα has no predicted N-glycosylation sites.

[0292] Human GPIbα contains sulfated tyrosines implicated in binding VWF within an acidic loop just C-terminal to the sequence included in the crystal structures. Murine GPIbα has a predicted sulfation site in the same loop, so that the differential binding of human vs. murine GPIbα to VWF-A1 is also likely to be small. The interfacial regions are otherwise highly conserved between species, with the exception of three salt bridges (See FIGS. **37**C-G). The conformation of the β-switch region is highly constrained, as noted in the main text. The only change to a buried interfacial residue is M239T (human to mouse), which lies in an invariant pocket. Notably, the crystal structure of the human "gain-of-function" mutant, M239V, shows no perturbations in this region, and given that valine is isosteric with threonine, this species difference is unlikely to affect either the complex structure or interspecies binding.

[0293] We used a consensus model of the human complex to build the murine model. We first overlaid murine A1 onto human A1 by fitting the central β-sheets (RMSD 0.3 Å; within experimental error); the only notable difference is the location of helix α4, which is shifted by 2-3 Å away from the GPIbα interface in the mouse owing to a larger residue on the buried face of this helix. For the GPIbα model, only the side-chains were altered, since the human and murine LRRs have identical lengths. Consensus rotamers with minimal steric clashes were chosen, followed by manual adjustment where necessary to create sensible van der waals interactions and H-bonding, using TURBOFRODO (Bio-Graphics, Marseille, France). Molecular overlays were optimized using LSQKAB (Collaborative Computational Project, No. 4. *Acta Crystallogr.* D50, 760-763 (1994)); molecular figures were created using MOLSCRIPT (Esnouf, R. M. *J. Mol. Graph. Model.* 15, 132-136 (1997)) and OPENGL (http://www.rush3d.com/reference/opengl-bluebook-1.0)

[0294] Statistics. An unpaired, two-tailed Student t test was used for multiple comparisons.

[0295] Results and Discussion

[0296] As the interaction between GPIbα and VWF-A1 is a prerequisite for effective thrombus formation in the arterial circulation, we first tested the ex vivo ability of surface-bound murine plasma VWF or its recombinant A1 domain (rVWF-A1) to support human platelet adhesion under physiologically relevant flow conditions: that is at a shear rate exceeding 1000 s^{-1} using a parallel-plate flow chamber (Ruggeri, Z. M. et al., (2006) *Blood.* 108, 1903-1910). The adhesive properties of VWF are tightly regulated such that it preferentially binds to platelets only when immobilized to sites of vascular injury and under hydrodynamic conditions encountered on the arterial side of the circulation (Sakariassen et al., (1979) *Nature* 279, 636-638; Ruggeri, Z. M. et al., (2006) *Blood.* 108, 1903-1910). Perfusion of human whole blood over murine plasma VWF or rVWF-A1 resulted in limited platelet deposition (10 to 25%, respectively) as compared with same-species controls (FIG. **36**A-B). Similarly, human VWF proteins had a diminished capacity to support murine platelet accumulation under identical conditions (FIGS. **36**C-D). This interspecies incompatibility would seem to preclude the study of human platelet behavior in a mouse model of arterial thrombosis.

[0297] In order to gain insight into the structural origins of this species incompatibility, we built models of murine-murine and human-murine GPIbα-VWF-A1 complexes based on the crystal structures of the human complex (Fukuda, K., et al., *Nat. Struct. Mol. Biol.* 12, 152-159 (2005); Dumas, J. J. et al., *J. Biol. Chem.* 279, 23327-23334 (2004); Huizinga, E. G. et al., *Science* 297, 1176-1179 (2002)) and human and murine VWF-A1 (Fukuda, K., et al., *Nat. Struct. Mol. Biol.* 12, 152-159 (2005)) (FIG. **37**A-E; see Methods).

[0298] The A1 domain comprises a Rossmann-like fold with a central, mostly parallel β-sheet flanked on both sides by α-helices (Fukuda, K., et al., *Nat. Struct. Mol. Biol.* 12, 152-159 (2005)). Human and murine VWF-A1 share considerable sequence (86% identity) and structure homology; in fact, the β-sheets of both species are identical within experimental error (a root mean square difference of 0.33 Å for Cα-atoms). The only major difference is the location of helix α4 (nomenclature as previously described in Dumas, J. J. et al., *J. Biol. Chem.* 279, 23327-23334 (2004)), which is shifted 2-3 Å away from the GPIbα binding site in the mouse, owing to a difference in a buried hydrophobic residue (FIG. **37**A-B). Although neither the structure of murine GPIbα nor its complex with the VWF-A1 domain are known, the high sequence similarity of the murine and human proteins (including the complex interface), as well as the rigid architecture of the leucine-rich repeats (LRR) of GPIbα, provide high confidence that their 3D structures will be highly homologous.

[0299] In the complexes, the major contact region involves the "β-switch" region (residues 227 to 241 in the C-terminal flank of GPIb alpha), which forms a β-hairpin that augments the β-sheet of the VWF-A1 domain. On its other side, this region of GPIbα packs tightly against the concave face of the LRR, which highly constrains it movement. Residues in mouse and human are mostly invariant on both sides of this interface. Notable exceptions are at position 1326 in VWF-A1, which is histidine (H) in humans versus an arginine (R) in mouse, and at position 238 in GPIb alpha, which is alanine (A) in humans versus an aspartic acid (D) in mouse (FIGS. **37**C and **37**D). A model of the murine complex suggests that these changes are complementary, since D238 can form an intermolecular salt-bridge with R1326; D238 in murine GPIb alpha also shields the positively charged flanking lysine (K) at position 231 (a conserved residue in both species) from unfavorable interactions with R1326 in murine VWF-A1. This salt-bridge cannot form in the human complex due to the presence of a histidine at 1326. However, an intermolecular salt-bridge can occur between R1395 and E225 located at the top of the human complex, which may compensate for this loss (FIG. **37**D). No such interaction can occur in the murine complex (FIG. **37**C).

[0300] In the human GPIb alpha-murine VWF-A1 interspecies complex, we predict that the two positively charged residues (GPIb alpha K231 and VWF-A1 R1326) create an electrostatic clash that impedes binding, owing to the lack of a negatively charged group at position 238 (FIG. **37**E). In the murine GPIb alpha-human VWF-A1 interspecies complex, however, no such electrostatic clash occurs despite the absence of the salt-bridge. There is, however, an overall change in net charge in the binding interface compared with the murine GPIb alpha-murine VWF-A1 complex (FIG. **37**F). This, together with the loss of critical salt-bridges, most likely accounts for the reduced interaction between mouse platelets and human VWF (See Tables 5 and 6).

TABLE 5

Predicted effect of species differences in residues on the human GPIb alpha-murine VWF-A1 interspecies complex.

mVWF-A1	hGPIb-alpha partner	hGPIb alpha-mVWF-A1	Reason
R1326	A238	(−)	Permits electrostatic clash of R1326 with K231 in GPIb alpha
E1330	K237	(+)	New salt-bridge E1330-K237
G1370	none	0	No interactions
R1395 (shifts position)	E225	(−)	Loss of salt-bridge

(+) = net positive,
(−) = net negative,
0 = minimal effect compared with syngeneic complexes.

TABLE 6

Predicted effect of species differences in residues on the murine GPIb alpha-human VWF-A1 interspecies complex.

hVWF-A1	mGPIb-alpha partner	hGPIb-alpha-mVWF-A1	Reason
H1326	D238	(−)	Loss of R1326-D238 salt-bridge
G1330	K237	(−)	Loss of E1330-K237 salt-bridge
S1370	none	0	No interactions
R1395 (shifts position)	N225	(+)	New polar interactions with R1395

(+) = net positive,
(−) = net negative,
0 = minimal effect compared with syngeneic complexes.

[0301] To explore the importance of the electrostatic mismatches in destabilizing the interspecies complexes, we substituted human residues into murine rVWF-A1 at positions 1326 (R>H), 1330 (E>G), and 1370 (S>G), and analyzed the ability of the mutant proteins to support human platelet accumulation under flow. As expected, amino acid substitutions at positions 1330 (predicted to remove a salt-bridge) and 1370 (predicted to have no effect) failed to promote the interaction between murine rVWF-A1 and human GPIbα. However, the 1326R>H mutation, which eliminates the electrostatic clash with K231, rendered murine A1 capable of supporting interactions at a level comparable to its wild-type (WT) human counterpart (FIG. **37**G). Similarly, conversion of 1326H>R in the human rVWF-A1 protein promoted the binding of mouse platelets, while the reverse substitution in its murine counterpart reduced adhesion by 75%. That a single residue change is sufficient for shifting the binding preferences across species supports the notion that this contact region is a "hot-spot" in the protein interface (Bogan, A. A. & Thorn, K. S. *J. Mol. Biol.* 280, 1-9 (1998)).

[0302] In order to determine the ability of full-length murine VWF containing the 1326R>H mutation ($VWF^{1326R>H}$) to support human platelet interactions and ultimately thrombus formation in vivo, we genetically modified mice to express $VWF^{1326R>H}$ (FIG. **38**B-C). Both homozygous and heterozygous animals were viable, fertile, born at the expected Mendelian ratio, and had platelet counts comparable to WT littermate controls. Moreover, reverse transcription-PCR(RT-PCR) of lung tissue from mutant mice with primers specific for the A1, A2, and/or A3 domains of VWF amplified cDNAs of the correct size and of similar intensity as compared to WT littermate controls (FIG. **39**A). VWF antigen levels, Factor VIII function, as well as VWF multimer pattern in homozygous mutant plasma were found to be equivalent to WT controls (FIG. **39**B-C). These results indicate that VWF gene translation, transcription, and post-translational modifications were not perturbed by our targeting strategy. The ability of plasma VWF to bind to collagen was also not affected by the introduction of the point mutation.

[0303] Hemostasis relies on platelet adhesion and activation at sites of vascular injury, which ultimately results in the formation of a hemostatic plug. To demonstrate the importance of VWF-A1 in this process, we measured bleeding times for mice possessing the 1326R>H mutation by removing 1 cm of distal tail (FIG. **41**A). In contrast to their WT counterparts, the vast majority of homozygous $VWF^{1326R>H}$ mice were incapable of forming an effective hemostatic plug, as they continued to bleed profusely throughout the duration of the experiment (10 min). Moreover, a smaller but statistically significant increase in bleeding time was noted for heterozygous animals (1.9-fold compared with WT; P=0.0055). Thus, disruption of a single salt bridge between murine VWF-A1 and GPIbα is sufficient to impair hemostasis.

[0304] To gain insight into how the 1326R>H mutation alterations hemostasis, we evaluated murine platelet adhesion at sites of vascular damage in vivo. A laser-induced vascular injury model was utilized to initiate platelet deposition in arterioles located in the microcirculation of the cremaster muscle of WT and homozygous mutant mice (Furie, B. & Furie., B. C. (2005) *J. Clin. Invest.* 115, 3355-3362). Although $VWF^{1326R>H}$ animals can initially form thrombi that fill the vessel lumen, they rapidly dissipated under the prevailing hydrodynamic conditions (FIG. **40**). By contrast, thrombi in WT mice continue to enlarge and eventually occlude blood flow under identical conditions.

[0305] To demonstrate that the removal of the electrostatic clash between residues 1326 and 231 in murine VWF-A1 and human GPIb alpha, respectively, promotes substantial interactions between this chimeric receptor-ligand pair, we perfused human whole blood over surface-immobilized plasma VWF obtained from mice homozygous for $VWF^{1326R>H}$ Remarkably, mutant murine VWF bound human platelets at levels comparable with its human counterpart (FIG. **41**B). Moreover, a function-blocking antibody 6D1, which binds exclusively to human GPIbα at the fourth LRR (Shen, Y. et al. (2000) *Blood.* 95, 903-910), inhibited platelet adhesion, demonstrating a key role for the platelet receptor in adhesion to $VWF^{1326R>H}$.

[0306] By contrast, the antibody SZ2 that recognizes the anionic-sulfated tyrosine sequence of GPIbα (residues 276 to 282) had a minimal affect on platelet accumulation, results consistent with a previous report (Fredrickson et al., (1998) *Blood.* 92, 3684-3693). The A1 domain also possesses the ability to support the movement of attached platelets in the direction of the prevailing hydrodynamic force owing to rapid and reversible interactions with GPIbα (Savage et al., (1996) *Cell* 84, 289-297; Doggett, T. A. et al. (2002) *Biophys. J.* 83, 194-205). We therefore compared translocation velocities of human platelets on either human or mutant murine VWF. Translocation velocities of human platelets on either homozygous mutant murine or native human plasma VWF were also similar (3.5±0.1 μm/sec vs. 3.2±0.1 μm/sec, respec-

tively; mean±s.e.m, n=4), demonstrating that $VWF^{1326R->H}$ functions in a manner indistinguishable from its human counterpart.

[0307] We next tested the ability of murine $VWF^{1326R>H}$ to support human platelet adhesion in vivo. The ability of human platelets to preferentially support thrombus formation was monitored simultaneously by labeling purified human cells with BCECF ex-vivo, and mouse platelets with rhodamine 6G by intravenous administration. Fluorescently-labeled human platelets were infused continuously via a catheter inserted into the femoral artery, resulting in a high local concentration of these cells within the microcirculation of the cremaster muscle. Their behavior in response to laser-induced vascular injury was monitored in real-time using confocal intravital microscopy (Furie, B. & Furie., B. C. (2005) *J. Clin. Invest.* 115, 3355-3362). Upon induction of laser damage to the vessel wall of arterioles in mice homozygous for $VWF^{1326R>H}$ human platelets rapidly adhered to the site of injury, forming large thrombi composed mainly of human cells (91.7±1.2%; mean±s.e.m) (FIG. **41**C-D); average thrombus size was 8,950±1,620 μm^2 (mean±s.e.m.). In WT mice, by contrast, human platelets had only a limited capacity to bind to the damaged vessel wall, accounting for only 5.7±0.4% of total thrombus area (mean±s.e.m.) (FIG. **41**C-D).

[0308] Consistent with the critical role of platelet GPIb alpha in mediating interactions with VWF-A1, pre-treatment of human platelets with mAb 6D1 greatly reduced thrombus size in the vasculature of $VWF^{1326R>H}$ mice (265±125 μm^2; mean±s.e.m.) (FIG. **41**E). This was also validated by our observations that human platelet deposition at sites of arterial injury is limited in VWF-deficient mice (185±35 μm^2; mean±s.e.m.), demonstrating that the A1 domain of this plasma protein serves as the major ligand for GPIbα in our humanized animal model of thrombosis.

[0309] Although GPIbα initiates platelet deposition at arterial shear rates, it is ultimately the platelet integrin αIIbβ3 that supports thrombus growth by promoting platelet-platelet interactions. The contribution of human αIIbβ3 in this process is demonstrated by the ability of the function blocking antibody 7E3 to also limit thrombus size (529±150 μm^2) (FIG. **41**E). Confirmation that the interaction formed between human platelets and murine $VWF^{1326R>H}$ is sufficient to promote effective hemostasis is provided by the ability of infused human cells to restore bleeding times in mutant mice to a level observed for their WT counterparts (182±14.5 s vs. 131.5±11.2 s, respectively; mean±s.e.m.) (FIG. **41**F). Importantly, human platelet-induced hemostatic clot formation can be completely disrupted in these animals by the preadministration of either clopidogrel (PLAVIX), an inhibitor of ADP-induced platelet activation, or abciximab (ReoPro®), a Fab fragment of the chimeric human-mouse monoclonal antibody 7E3, which blocks the function of αIIbβ3 on human but not murine platelets (Hankey, G. J. & Eikelboom, J. W. Antiplatelet drugs. *Med. J. Aust.* 178, 568-574; Bennett, J. S. (2001) *Annu. Rev. Med.* 52, 161-184) (FIG. **41**F).

[0310] In summary, these studies demonstrate how one can effectively utilize atomic models of interspecies complexes to identify a binding hot spot where a disproportionate amount of the binding free energy is localized, such that a single amino acid substitution significantly affects the interaction (Bogan, A. A. & Thorn, K. S. (1998) *J. Mol. Biol.* 280, 1-9), and in this case switches species specificity. Moreover, a subtle and localized change of this nature limits the possibility of inducing structural perturbations that impact on the function of other domains contained within VWF. These show that human platelet adhesion to $VWF^{1326R>H}$ is dependent on GPIbα binding to VWF-A1, with other potential ligands for this receptor playing a subservient role in this process (Bergmeier, W. et al. (2006) *Proc. Natl. Acad. Sci. USA.* 103, 16900-16905). The reliance of human thrombus formation on the integrin αIIbβ3, as well as the ability of the FDA approved drugs PLAVIX and ReoPro® to impair human platelet-mediated hemostasis indicate that downstream adhesive and activation events known to be critical for clot formation and stability are intact in the mutant VWF animals. Thus, we anticipate that the $VWF^{1326R>H}$ knock-in mice will prove useful in the preclinical evaluation of new antithrombotic therapeutics designed ultimately for human use. These results also have implications for advancing both knowledge of human platelet biology and in preclinical testing of antithrombotic therapies in vivo.

Example 5

Use of "Humanized" VWF-A1 Animal for Developing Technologies to Image Sites of Occult Bleeding or Thrombus Formation in Humans

[0311] Perfluorocarbon Nanoparticle based imaging platform. The ability of a VWF-A1 mutant animal, such as our 1326R>H mutant mouse, to generate thrombi composed of human platelets at sites of vascular injury in vivo, provides a means for developing imaging technologies designed to detect sites of occult bleeding or thrombus formation in humans. For example, such technologies may prove useful expediting the discovery of sites of internal bleeding in humans as a result of injuries obtained form a motor vehicle accident. Similarly, it may be useful in detecting injuries obtained in a military battle. Suitable probes include antibodies, small molecules, peptides that recognize molecules expressed on human platelets or the various domains of VWF. However, coupling contrast agents directly to antibodies is cumbersome and insufficient for detection of such complexes in the body by various imaging modalities (i.e. MRI) due to low signal to noise output. Thus, an ideal candidate for detection would not only preserve the specificity associated with monoclonal antibodies, small molecules, or peptides but also have the following properties: 1) high signal-to-noise ratio, 2) long circulating half-life, 3) acceptable toxicity profile, 4) ease of use and production, and 5) compatibility with standard commercially available imaging modalities. Perfluorocarbon Nanoparticle (PNP) may provide the answer. This proposal will take advantage of a novel nanoparticle contrast agent that can be imaged by ultrasound, magnetic resonance, and nuclear imaging (Lanza et al., (2000) *Invest Radiol,* 35: 227-234; Lanza et al., (1997) *Ultrasound Med Biol,* 23: 863-870; Yu et al., (2000) *Magn Reson Med.* 44(6):867-72). This agent is a small (~150-250 nanometer diameter), lipid encapsulated, perfluorocarbon emulsion that can be administered by vein. Importantly, monoclonal antibodies as well as small molecules and peptides that recognize platelets and/or VWF can be covalently coupled to PNPs. Moreover, PNPs can also be potentially used for targeted drug delivery (FIG. **42**).

[0312] PNPs have been shown to remain stable in the circulation with a half-life of >1 hour, which permits rapid binding and local contrast enhancement sufficient for diagnostic imaging within 30-60 minutes. PNPs are cleared by the liver and spleen, and are similar to "artificial blood" formulations used to enhance oxygen, which have acceptable safety

profiles for clinical use at 10 times greater dose than would be required for targeted contrast enhancement. In addition, perfluorocarbon to be used in this study (perfluorooctylbromide) has an extensive tract record for human safety in clinical trials (i.e. Oxygent, Alliance Pharmaceuticals). Thus, this nanoparticle platform provides an ideal opportunity to prove that contrast agents can be targeted specifically to sites of human thrombus formation.

[0313] Preparation of Fluorescently-Labeled Antibody Targeted Nanoparticles.

[0314] The basic method for formulating perfluorocarbon nanoparticles comprised of perfluorooctyl bromide (40% w/v), a surfactant co-mixture (2.0%, w/v) and glycerin 9(1.7%, w/v) has been well described (Lanza et al., (2000) *Invest Radiol,* 35: 227-234; Lanza et al., (1997) *Ultrasound Med Biol,* 23: 863-870).

[0315] Briefly, the surfactant co-mixture is dissolved in chloroform/methanol, evaporated under reduced pressure, dried in a 50° C. vacuum oven, and finally dispersed into water by sonication. The suspension is combined with perfluorocarbon and then emulsified at 20,000 PSI. Fluorescent nanoparticles are manufactured by including in the lipid mixture 0.1 mole % Fluorescence-FITC or PE prior to the emulsification step. Coupling of monoclonal antibodies involves the introduction of a sulflhydrl group onto the protein by modification of amines with N-succinimidyl S-acetylthioacetate (SATA), which then is reacted with nanoparticles containing activated maleimide. We coupled an antibody that recognizes the human, but not mouse, platelet receptor alphaIIb $beta_3$ and determined the ability of FITC-labeled PNPs to detect a thrombus composed of human platelets at a site of laser-induced vascular injury in the cremaster muscle of a mouse homozygous for the 1326R>H mutation. These antibody-coupled PNPs rapidly and selectively accumulated at the site of the developing human thrombus (FIG. **43**).

Example 6

Identification of Small Molecules that Mitigate Binding Between GPIb Alpha and the VWF-A1 Domain

[0316] Small molecules, often with molecular weights of 500 or below, have proven to be extremely important to researchers to explore function at the molecular, cellular, and in vivo level. Such compounds have also been proven to be valuable as drugs to treat diseases, and most medicines marketed today are from this class (i.e. Aggrastat—see above). As the interaction between GPIb alpha and VWF-A1 is essential for the platelet deposition in damaged arterioles, it is a reasonable to assume that disruption of this adhesive event will inhibit or ameliorate thrombus formation. Moreover, we speculate that only partial inhibition is required to achieve this goal based on the phenotype of our mutant A1 domain mice, the inability to form stable thrombi in vivo.

[0317] Computational design based on the structure of the binary complex. Traditional approaches to small molecule discovery typically rely on a step-wise synthesis and screening program for large numbers of compounds to optimize activity profiles. Over the past decade, scientists have used computer models to aid in the development of new chemical agonists or antagonists as well as to better define activity profiles and binding affinities of such compounds. In particular, these tools are being successfully used, in conjunction with traditional research techniques, to examine the structural properties of existing compounds in order to predict their ability to alter the function of biologically relevant proteins. For this approach to be successful, one must have high quality crystal structures of the biological molecule(s) in order to generate an accurate 3-dimensional model so that it can then be used to identify binding regions for small molecules.

[0318] The structure of the binary complex formed when GPIb alpha binds to the A1 domain of VWF can be determined using such methods. For example, a mechanism by which the snake venom protein botrocetin enhances the interaction between GPIb alpha and the VWF-A1 in order to promote spontaneous platelet aggregation, resulting in death has been elucidated. Botrocetin was known to bind with high affinity to the A1 domain [see Table 7 for the crystallization data summary and Table 8 for atomic coordinate data], but was not thought to interact directly with GPIb alpha. This snake venom has the capacity to form a small, but distinct interface with this platelet receptor so to prevent its release from the A1 domain, thus facilitating platelet aggregation (FIG. **44**). In a sense, nature has created a molecule that modifies the behavior of a known biological interaction, suggesting that one may be able to target man-made structures to this domain as well.

TABLE 7

Summary of Crystallization data (from PDB access no. 1AUQ and J Biol Chem (1998) 273 (17): 10396-10401)

Structure	Free enzyme (see Table 8)
Space Group	$P6_1$
Maximum resolution (Å)	2.3
Resolution range for refinement	10-2.3
Number of reflections	11,849
Completeness (%)	85.4
R factor^2 (%)	18.6
Free R factor^2 (%)	23.8
rms deviation in bond lengths (Å)	0.011
rms deviation in bond angles (°)	1.43

TABLE 8

Atomic Coordinates for Residues of a Crystal of murine VWF-A1 (SEQ ID NO: 10).

CRYST1	86.395	86.395	68.125	90.00	90.00	120.00	P 61			6
ATOM	1	N	ASP	498	22.142	52.453	−14.520	1.00	83.59	N
ATOM	2	CA	ASP	498	20.770	52.768	−14.026	1.00	83.86	C
ATOM	3	C	ASP	498	20.803	53.068	−12.522	1.00	81.16	C
ATOM	4	O	ASP	498	20.978	52.165	−11.696	1.00	81.93	O
ATOM	5	CB	ASP	498	19.821	51.597	−14.327	1.00	87.23	C
ATOM	6	CG	ASP	498	18.352	51.961	−14.133	1.00	88.94	C
ATOM	7	OD1	ASP	498	18.022	53.171	−14.084	1.00	88.91	O

TABLE 8-continued

Atomic Coordinates for Residues of a Crystal of murine VWF-A1 (SEQ ID NO: 10).

ATOM	8	OD2	ASP	498	17.521	51.027	−14.033	1.00	90.11	O
ATOM	9	N	ILE	499	20.620	54.343	−12.190	1.00	76.43	N
ATOM	10	CA	ILE	499	20.649	54.829	−10.809	1.00	70.82	C
ATOM	11	C	ILE	499	19.237	55.126	−10.255	1.00	64.40	C
ATOM	12	O	ILE	499	18.888	56.265	−9.941	1.00	62.53	O
ATOM	13	CB	ILE	499	21.596	56.076	−10.724	1.00	75.17	C
ATOM	14	CG1	ILE	499	21.872	56.476	−9.278	1.00	77.18	C
ATOM	15	CG2	ILE	499	21.030	57.256	−11.527	1.00	77.54	C
ATOM	16	CD1	ILE	499	23.010	57.485	−9.141	1.00	79.34	C
ATOM	17	N	SER	500	18.445	54.071	−10.111	1.00	58.81	N
ATOM	18	CA	SER	500	17.073	54.170	−9.616	1.00	52.67	C
ATOM	19	C	SER	500	16.771	52.914	−8.797	1.00	46.03	C
ATOM	20	O	SER	500	17.477	51.915	−8.928	1.00	46.10	O
ATOM	21	CB	SER	500	16.085	54.306	−10.779	1.00	55.03	C
ATOM	22	OG	SER	500	14.814	54.747	−10.320	1.00	55.43	O
ATOM	23	N	GLU	501	15.690	52.946	−8.017	1.00	39.21	N
ATOM	24	CA	GLU	501	15.341	51.839	−7.124	1.00	33.62	C
ATOM	25	C	GLU	501	13.826	51.632	−6.967	1.00	33.73	C
ATOM	26	O	GLU	501	13.055	52.575	−7.148	1.00	35.42	O
ATOM	27	CB	GLU	501	16.025	52.088	−5.772	1.00	27.09	C
ATOM	28	CG	GLU	501	15.928	53.517	−5.328	1.00	23.81	C
ATOM	29	CD	GLU	501	17.036	53.971	−4.394	1.00	22.26	C
ATOM	30	OE1	GLU	501	18.150	53.442	−4.407	1.00	23.46	O
ATOM	31	OE2	GLU	501	16.790	54.912	−3.645	1.00	19.78	O
ATOM	32	N	PRO	502	13.390	50.408	−6.585	1.00	32.77	N
ATOM	33	CA	PRO	502	11.980	50.038	−6.406	1.00	33.37	C
ATOM	34	C	PRO	502	11.257	50.886	−5.377	1.00	36.76	C
ATOM	35	O	PRO	502	11.881	51.657	−4.655	1.00	40.35	O
ATOM	36	CB	PRO	502	12.056	48.588	−5.911	1.00	32.84	C
ATOM	37	CG	PRO	502	13.426	48.154	−6.188	1.00	30.80	C
ATOM	38	CD	PRO	502	14.248	49.372	−5.994	1.00	33.53	C
ATOM	39	N	PRO	503	9.921	50.743	−5.283	1.00	38.50	N
ATOM	40	CA	PRO	503	9.095	51.495	−4.332	1.00	36.00	C
ATOM	41	C	PRO	503	9.217	50.861	−2.962	1.00	33.78	C
ATOM	42	O	PRO	503	9.351	49.642	−2.870	1.00	33.98	O
ATOM	43	CB	PRO	503	7.670	51.270	−4.844	1.00	35.94	C
ATOM	44	CG	PRO	503	7.844	50.708	−6.240	1.00	36.76	C
ATOM	45	CD	PRO	503	9.074	49.889	−6.137	1.00	37.21	C
ATOM	46	N	LEU	504	9.225	51.670	−1.910	1.00	32.48	N
ATOM	47	CA	LEU	504	9.284	51.120	−0.562	1.00	31.29	C
ATOM	48	C	LEU	504	7.869	50.821	−0.120	1.00	31.74	C
ATOM	49	O	LEU	504	7.663	50.104	0.847	1.00	34.42	O
ATOM	50	CB	LEU	504	9.857	52.133	0.427	1.00	27.31	C
ATOM	51	CG	LEU	504	11.346	52.216	0.633	1.00	20.47	C
ATOM	52	CD1	LEU	504	11.608	53.354	1.577	1.00	16.31	C
ATOM	53	CD2	LEU	504	11.865	50.886	1.213	1.00	22.70	C
ATOM	54	N	HIS	505	6.892	51.433	−0.779	1.00	32.33	N
ATOM	55	CA	HIS	505	5.508	51.234	−0.382	1.00	33.59	C
ATOM	56	C	HIS	505	4.574	50.872	−1.506	1.00	37.41	C
ATOM	57	O	HIS	505	4.980	50.817	−2.669	1.00	38.66	O
ATOM	58	CB	HIS	505	4.977	52.504	0.277	1.00	30.39	C
ATOM	59	CG	HIS	505	5.799	52.956	1.432	1.00	24.50	C
ATOM	60	ND1	HIS	505	6.533	54.118	1.413	1.00	26.88	N
ATOM	61	CD2	HIS	505	6.041	52.377	2.628	1.00	22.75	C
ATOM	62	CE1	HIS	505	7.195	54.235	2.543	1.00	22.86	C
ATOM	63	NE2	HIS	505	6.915	53.193	3.297	1.00	23.78	N
ATOM	64	N	ASP	506	3.323	50.612	−1.125	1.00	39.57	N
ATOM	65	CA	ASP	506	2.254	50.291	−2.072	1.00	41.22	C
ATOM	66	C	ASP	506	1.600	51.614	−2.482	1.00	39.47	C
ATOM	67	O	ASP	506	1.306	51.826	−3.651	1.00	42.48	O
ATOM	68	CB	ASP	506	1.231	49.325	−1.443	1.00	44.20	C
ATOM	69	CG	ASP	506	1.831	47.941	−1.106	1.00	47.68	C
ATOM	70	OD1	ASP	506	2.837	47.508	−1.730	1.00	48.34	O
ATOM	71	OD2	ASP	506	1.276	47.272	−0.209	1.00	49.92	O
ATOM	72	N	PHE	507	1.367	52.497	−1.507	1.00	38.50	N
ATOM	73	CA	PHE	507	0.800	53.830	−1.756	1.00	35.03	C
ATOM	74	C	PHE	507	2.016	54.527	−2.406	1.00	36.00	C
ATOM	75	O	PHE	507	3.086	54.637	−1.780	1.00	35.29	O
ATOM	76	CB	PHE	507	0.408	54.471	−0.418	1.00	32.75	C
ATOM	77	CG	PHE	507	−0.252	55.810	−0.552	1.00	32.71	C
ATOM	78	CD1	PHE	507	−1.605	55.915	−0.866	1.00	33.60	C
ATOM	79	CD2	PHE	507	0.471	56.969	−0.343	1.00	29.61	C
ATOM	80	CE1	PHE	507	−2.216	57.157	−0.962	1.00	31.49	C

TABLE 8-continued

Atomic Coordinates for Residues of a Crystal of murine VWF-A1 (SEQ ID NO: 10).

ATOM	81	CE2	PHE	507	−0.132	58.205	−0.437	1.00	29.32	C
ATOM	82	CZ	PHE	507	−1.477	58.298	−0.750	1.00	33.05	C
ATOM	83	N	TYR	508	1.839	55.032	−3.624	1.00	35.32	N
ATOM	84	CA	TYR	508	2.955	55.578	−4.396	1.00	32.82	C
ATOM	85	C	TYR	508	2.445	56.499	−5.515	1.00	33.01	C
ATOM	86	O	TYR	508	1.484	56.154	−6.200	1.00	39.06	O
ATOM	87	CB	TYR	508	3.612	54.364	−5.053	1.00	27.12	C
ATOM	88	CG	TYR	508	5.027	54.464	−5.529	1.00	31.57	C
ATOM	89	CD1	TYR	508	6.041	54.922	−4.680	1.00	35.35	C
ATOM	90	CD2	TYR	508	5.389	53.946	−6.771	1.00	28.70	C
ATOM	91	CE1	TYR	508	7.397	54.848	−5.054	1.00	36.51	C
ATOM	92	CE2	TYR	508	6.730	53.862	−7.154	1.00	33.58	C
ATOM	93	CZ	TYR	508	7.735	54.309	−6.294	1.00	36.83	C
ATOM	94	OH	TYR	508	9.075	54.176	−6.649	1.00	40.00	O
ATOM	95	N	CYS	509	3.046	57.662	−5.717	1.00	28.11	N
ATOM	96	CA	CYS	509	2.593	58.516	−6.822	1.00	27.15	C
ATOM	97	C	CYS	509	3.509	58.310	−8.009	1.00	25.32	C
ATOM	98	O	CYS	509	4.717	58.512	−7.909	1.00	30.67	O
ATOM	99	CB	CYS	509	2.572	59.997	−6.464	1.00	25.77	C
ATOM	100	SG	CYS	509	1.888	61.004	−7.834	1.00	21.50	S
ATOM	101	N	SER	510	2.939	57.837	−9.102	1.00	23.50	N
ATOM	102	CA	SER	510	3.686	57.590	−10.321	1.00	25.91	C
ATOM	103	C	SER	510	3.052	58.414	−11.475	1.00	25.38	C
ATOM	104	O	SER	510	2.384	57.882	−12.364	1.00	32.01	O
ATOM	105	CB	SER	510	3.687	56.087	−10.612	1.00	24.85	C
ATOM	106	OG	SER	510	4.275	55.783	−11.867	1.00	29.60	O
ATOM	107	N	ARG	511	3.236	59.726	−11.434	1.00	19.35	N
ATOM	108	CA	ARG	511	2.689	60.596	−12.451	1.00	16.16	C
ATOM	109	C	ARG	511	3.757	61.589	−12.810	1.00	20.03	C
ATOM	110	O	ARG	511	4.825	61.584	−12.191	1.00	24.48	O
ATOM	111	CB	ARG	511	1.439	61.310	−11.952	1.00	13.51	C
ATOM	112	CG	ARG	511	0.300	60.358	−11.708	1.00	13.74	C
ATOM	113	CD	ARG	511	−0.994	61.084	−11.674	1.00	12.03	C
ATOM	114	NE	ARG	511	−2.111	60.169	−11.505	1.00	12.96	N
ATOM	115	CZ	ARG	511	−3.388	60.547	−11.582	1.00	16.84	C
ATOM	116	NH1	ARG	511	−3.700	61.814	−11.841	1.00	18.71	N
ATOM	117	NH2	ARG	511	−4.361	59.680	−11.342	1.00	18.41	N
ATOM	118	N	LEU	512	3.482	62.427	−13.810	1.00	16.95	N
ATOM	119	CA	LEU	512	4.457	63.400	−14.279	1.00	17.28	C
ATOM	120	C	LEU	512	4.337	64.645	−13.434	1.00	16.82	C
ATOM	121	O	LEU	512	3.544	65.534	−13.715	1.00	18.14	O
ATOM	122	CB	LEU	512	4.220	63.668	−15.756	1.00	18.43	C
ATOM	123	CG	LEU	512	4.357	62.372	−16.556	1.00	17.02	C
ATOM	124	CD1	LEU	512	3.947	62.602	−17.993	1.00	15.13	C
ATOM	125	CD2	LEU	512	5.796	61.865	−16.461	1.00	16.19	C
ATOM	126	N	LEU	513	5.226	64.748	−12.458	1.00	19.32	N
ATOM	127	CA	LEU	513	5.162	65.827	−11.501	1.00	18.08	C
ATOM	128	C	LEU	513	6.506	66.390	−11.089	1.00	15.12	C
ATOM	129	O	LEU	513	7.477	65.655	−10.948	1.00	14.11	O
ATOM	130	CB	LEU	513	4.454	65.273	−10.257	1.00	19.36	C
ATOM	131	CG	LEU	513	4.260	66.123	−9.012	1.00	20.52	C
ATOM	132	CD1	LEU	513	3.338	67.259	−9.353	1.00	18.37	C
ATOM	133	CD2	LEU	513	3.698	65.270	−7.902	1.00	12.16	C
ATOM	134	N	ASP	514	6.534	67.699	−10.853	1.00	16.07	N
ATOM	135	CA	ASP	514	7.735	68.384	−10.383	1.00	16.17	C
ATOM	136	C	ASP	514	7.257	68.946	−9.041	1.00	15.61	C
ATOM	137	O	ASP	514	6.480	69.890	−9.014	1.00	16.76	O
ATOM	138	CB	ASP	514	8.136	69.530	−11.327	1.00	15.99	C
ATOM	139	CG	ASP	514	9.016	69.076	−12.511	1.00	18.33	C
ATOM	140	OD1	ASP	514	9.255	67.874	−12.736	1.00	17.22	O
ATOM	141	OD2	ASP	514	9.492	69.961	−13.240	1.00	25.18	O
ATOM	142	N	LEU	515	7.660	68.338	−7.929	1.00	15.44	N
ATOM	143	CA	LEU	515	7.205	68.808	−6.623	1.00	14.64	C
ATOM	144	C	LEU	515	8.282	69.524	−5.802	1.00	14.01	C
ATOM	145	O	LEU	515	9.382	68.995	−5.603	1.00	8.40	O
ATOM	146	CB	LEU	515	6.613	67.625	−5.853	1.00	14.99	C
ATOM	147	CG	LEU	515	6.168	67.788	−4.396	1.00	9.59	C
ATOM	148	CD1	LEU	515	5.054	68.775	−4.249	1.00	5.58	C
ATOM	149	CD2	LEU	515	5.714	66.455	−3.924	1.00	8.85	C
ATOM	150	N	VAL	516	7.975	70.743	−5.355	1.00	10.50	N
ATOM	151	CA	VAL	516	8.927	71.514	−4.566	1.00	13.88	C
ATOM	152	C	VAL	516	8.504	71.601	−3.100	1.00	14.19	C
ATOM	153	O	VAL	516	7.400	72.059	−2.796	1.00	14.17	O

TABLE 8-continued

Atomic Coordinates for Residues of a Crystal of murine VWF-A1 (SEQ ID NO: 10).

ATOM	154	CB	VAL	516	9.103	72.966	−5.111	1.00	15.97	C
ATOM	155	CG1	VAL	516	10.134	73.711	−4.278	1.00	14.88	C
ATOM	156	CG2	VAL	516	9.580	72.947	−6.570	1.00	14.16	C
ATOM	157	N	PHE	517	9.359	71.127	−2.191	1.00	17.00	N
ATOM	158	CA	PHE	517	9.072	71.205	−0.747	1.00	13.51	C
ATOM	159	C	PHE	517	9.776	72.469	−0.179	1.00	12.08	C
ATOM	160	O	PHE	517	11.009	72.587	−0.233	1.00	10.17	O
ATOM	161	CB	PHE	517	9.602	69.968	−0.024	1.00	15.54	C
ATOM	162	CG	PHE	517	8.940	68.662	−0.426	1.00	11.63	C
ATOM	163	CD1	PHE	517	7.805	68.200	0.230	1.00	13.47	C
ATOM	164	CD2	PHE	517	9.517	67.852	−1.400	1.00	9.53	C
ATOM	165	CE1	PHE	517	7.256	66.952	−0.075	1.00	10.36	C
ATOM	166	CE2	PHE	517	8.989	66.625	−1.703	1.00	8.49	C
ATOM	167	CZ	PHE	517	7.847	66.164	−1.036	1.00	10.76	C
ATOM	168	N	LEU	518	9.005	73.405	0.378	1.00	12.33	N
ATOM	169	CA	LEU	518	9.574	74.643	0.926	1.00	12.65	C
ATOM	170	C	LEU	518	9.420	74.659	2.453	1.00	11.22	C
ATOM	171	O	LEU	518	8.333	74.940	2.959	1.00	15.25	O
ATOM	172	CB	LEU	518	8.884	75.865	0.287	1.00	9.93	C
ATOM	173	CG	LEU	518	8.791	75.956	−1.244	1.00	8.05	C
ATOM	174	CD1	LEU	518	7.840	77.076	−1.640	1.00	3.79	C
ATOM	175	CD2	LEU	518	10.166	76.182	−1.857	1.00	10.27	C
ATOM	176	N	LEU	519	10.514	74.399	3.168	1.00	7.04	N
ATOM	177	CA	LEU	519	10.518	74.319	4.635	1.00	7.57	C
ATOM	178	C	LEU	519	10.795	75.567	5.440	1.00	4.28	C
ATOM	179	O	LEU	519	11.867	76.131	5.339	1.00	10.10	O
ATOM	180	CB	LEU	519	11.544	73.283	5.090	1.00	6.92	C
ATOM	181	CG	LEU	519	11.188	71.821	4.925	1.00	10.92	C
ATOM	182	CD1	LEU	519	11.011	71.550	3.470	1.00	8.66	C
ATOM	183	CD2	LEU	519	12.295	70.951	5.489	1.00	7.00	C
ATOM	184	N	ASP	520	9.891	75.908	6.352	1.00	11.69	N
ATOM	185	CA	ASP	520	10.051	77.081	7.217	1.00	13.58	C
ATOM	186	C	ASP	520	11.330	76.914	8.057	1.00	14.38	C
ATOM	187	O	ASP	520	11.469	75.933	8.775	1.00	18.86	O
ATOM	188	CB	ASP	520	8.813	77.201	8.116	1.00	15.76	C
ATOM	189	CG	ASP	520	8.737	78.528	8.839	1.00	14.91	C
ATOM	190	OD1	ASP	520	9.744	78.970	9.394	1.00	15.53	O
ATOM	191	OD2	ASP	520	7.671	79.143	8.870	1.00	15.00	O
ATOM	192	N	GLY	521	12.272	77.847	7.958	1.00	12.62	N
ATOM	193	CA	GLY	521	13.514	77.720	8.713	1.00	12.88	C
ATOM	194	C	GLY	521	13.617	78.637	9.919	1.00	17.26	C
ATOM	195	O	GLY	521	14.690	78.785	10.508	1.00	16.89	O
ATOM	196	N	SER	522	12.500	79.256	10.291	1.00	16.08	N
ATOM	197	CA	SER	522	12.452	80.168	11.420	1.00	17.98	C
ATOM	198	C	SER	522	12.484	79.424	12.750	1.00	18.91	C
ATOM	199	O	SER	522	12.359	78.197	12.792	1.00	17.50	O
ATOM	200	CB	SER	522	11.158	80.967	11.371	1.00	12.88	C
ATOM	201	OG	SER	522	10.069	80.128	11.708	1.00	12.60	O
ATOM	202	N	SER	523	12.547	80.206	13.828	1.00	17.68	N
ATOM	203	CA	SER	523	12.558	79.701	15.187	1.00	20.71	C
ATOM	204	C	SER	523	11.141	79.389	15.694	1.00	20.97	C
ATOM	205	O	SER	523	10.971	78.999	16.833	1.00	27.46	O
ATOM	206	CB	SER	523	13.253	80.693	16.111	1.00	21.32	C
ATOM	207	OG	SER	523	12.701	81.986	15.933	1.00	26.32	O
ATOM	208	N	ARG	524	10.117	79.590	14.872	1.00	18.19	N
ATOM	209	CA	ARG	524	8.765	79.240	15.278	1.00	19.04	C
ATOM	210	C	ARG	524	8.655	77.734	15.460	1.00	20.87	C
ATOM	211	O	ARG	524	7.724	77.235	16.106	1.00	22.79	O
ATOM	212	CB	ARG	524	7.758	79.709	14.246	1.00	20.78	C
ATOM	213	CG	ARG	524	7.654	81.183	14.225	1.00	22.74	C
ATOM	214	CD	ARG	524	7.449	81.671	15.627	1.00	23.78	C
ATOM	215	NE	ARG	524	7.265	83.107	15.661	1.00	29.27	N
ATOM	216	CZ	ARG	524	7.116	83.815	16.774	1.00	29.96	C
ATOM	217	NH1	ARG	524	7.140	83.218	17.959	1.00	25.48	N
ATOM	218	NH2	ARG	524	6.899	85.118	16.686	1.00	26.64	N
ATOM	219	N	LEU	525	9.561	77.014	14.804	1.00	19.12	N
ATOM	220	CA	LEU	525	9.645	75.571	14.915	1.00	17.29	C
ATOM	221	C	LEU	525	10.942	75.381	15.674	1.00	22.24	C
ATOM	222	O	LEU	525	11.950	76.041	15.374	1.00	21.49	O
ATOM	223	CB	LEU	525	9.817	74.913	13.546	1.00	19.88	C
ATOM	224	CG	LEU	525	8.695	74.845	12.523	1.00	15.92	C
ATOM	225	CD1	LEU	525	9.236	74.241	11.253	1.00	11.38	C
ATOM	226	CD2	LEU	525	7.556	73.989	13.080	1.00	16.42	C

TABLE 8-continued

Atomic Coordinates for Residues of a Crystal of murine VWF-A1 (SEQ ID NO: 10).

ATOM	227	N	SER	526	10.912	74.519	16.682	1.00	23.55	N
ATOM	228	CA	SER	526	12.113	74.240	17.460	1.00	24.19	C
ATOM	229	C	SER	526	12.873	73.147	16.725	1.00	25.31	C
ATOM	230	O	SER	526	12.342	72.534	15.797	1.00	26.87	O
ATOM	231	CB	SER	526	11.737	73.746	18.855	1.00	22.27	C
ATOM	232	OG	SER	526	10.926	72.579	18.785	1.00	18.28	O
ATOM	233	N	GLU	527	14.093	72.868	17.160	1.00	26.48	N
ATOM	234	CA	GLU	527	14.900	71.829	16.539	1.00	26.18	C
ATOM	235	C	GLU	527	14.144	70.514	16.449	1.00	25.55	C
ATOM	236	O	GLU	527	14.134	69.859	15.406	1.00	30.55	O
ATOM	237	CB	GLU	527	16.172	71.620	17.349	1.00	28.26	C
ATOM	238	CG	GLU	527	17.025	70.471	16.880	1.00	37.14	C
ATOM	239	CD	GLU	527	18.417	70.909	16.459	1.00	44.14	C
ATOM	240	OE1	GLU	527	19.082	71.637	17.238	1.00	44.73	O
ATOM	241	OE2	GLU	527	18.853	70.506	15.351	1.00	47.92	O
ATOM	242	N	ALA	528	13.458	70.159	17.526	1.00	23.12	N
ATOM	243	CA	ALA	528	12.733	68.901	17.580	1.00	19.11	C
ATOM	244	C	ALA	528	11.547	68.875	16.650	1.00	19.81	C
ATOM	245	O	ALA	528	11.237	67.834	16.078	1.00	24.34	O
ATOM	246	CB	ALA	528	12.301	68.609	18.992	1.00	11.67	C
ATOM	247	N	GLU	529	10.873	70.015	16.518	1.00	19.26	N
ATOM	248	CA	GLU	529	9.697	70.126	15.659	1.00	19.04	C
ATOM	249	C	GLU	529	10.146	70.132	14.212	1.00	21.14	C
ATOM	250	O	GLU	529	9.491	69.543	13.358	1.00	26.65	O
ATOM	251	CB	GLU	529	8.908	71.403	15.974	1.00	17.25	C
ATOM	252	CG	GLU	529	8.144	71.355	17.292	1.00	12.54	C
ATOM	253	CD	GLU	529	7.447	72.663	17.624	1.00	18.73	C
ATOM	254	OE1	GLU	529	8.120	73.725	17.576	1.00	16.59	O
ATOM	255	OE2	GLU	529	6.236	72.623	17.966	1.00	18.64	O
ATOM	256	N	PHE	530	11.292	70.750	13.945	1.00	17.88	N
ATOM	257	CA	PHE	530	11.828	70.807	12.600	1.00	18.23	C
ATOM	258	C	PHE	530	12.139	69.383	12.147	1.00	21.70	C
ATOM	259	O	PHE	530	11.848	69.007	11.003	1.00	28.22	O
ATOM	260	CB	PHE	530	13.083	71.658	12.571	1.00	17.84	C
ATOM	261	CG	PHE	530	13.534	72.028	11.196	1.00	19.34	C
ATOM	262	CD1	PHE	530	12.696	72.758	10.342	1.00	17.07	C
ATOM	263	CD2	PHE	530	14.806	71.685	10.759	1.00	16.08	C
ATOM	264	CE1	PHE	530	13.127	73.139	9.089	1.00	12.46	C
ATOM	265	CE2	PHE	530	15.248	72.066	9.492	1.00	13.83	C
ATOM	266	CZ	PHE	530	14.413	72.789	8.664	1.00	11.80	C
ATOM	267	N	GLU	531	12.650	68.563	13.052	1.00	18.89	N
ATOM	268	CA	GLU	531	12.932	67.182	12.695	1.00	21.46	C
ATOM	269	C	GLU	531	11.660	66.478	12.263	1.00	17.63	C
ATOM	270	O	GLU	531	11.711	65.606	11.397	1.00	22.80	O
ATOM	271	CB	GLU	531	13.556	66.414	13.862	1.00	19.16	C
ATOM	272	CG	GLU	531	14.885	66.955	14.274	1.00	24.82	C
ATOM	273	CD	GLU	531	15.827	67.120	13.105	1.00	28.99	C
ATOM	274	OE1	GLU	531	16.177	66.101	12.469	1.00	24.87	O
ATOM	275	OE2	GLU	531	16.211	68.282	12.831	1.00	35.27	O
ATOM	276	N	VAL	532	10.529	66.840	12.864	1.00	15.71	N
ATOM	277	CA	VAL	532	9.257	66.211	12.509	1.00	15.96	C
ATOM	278	C	VAL	532	8.820	66.691	11.133	1.00	18.38	C
ATOM	279	O	VAL	532	8.234	65.921	10.357	1.00	22.15	O
ATOM	280	CB	VAL	532	8.149	66.493	13.551	1.00	15.96	C
ATOM	281	CG1	VAL	532	6.825	65.847	13.117	1.00	6.01	C
ATOM	282	CG2	VAL	532	8.575	65.950	14.908	1.00	13.74	C
ATOM	283	N	LEU	533	9.150	67.943	10.821	1.00	14.73	N
ATOM	284	CA	LEU	533	8.826	68.530	9.536	1.00	14.97	C
ATOM	285	C	LEU	533	9.602	67.771	8.476	1.00	16.19	C
ATOM	286	O	LEU	533	9.044	67.348	7.458	1.00	18.03	O
ATOM	287	CB	LEU	533	9.243	70.008	9.496	1.00	14.45	C
ATOM	288	CG	LEU	533	8.780	70.717	8.220	1.00	12.15	C
ATOM	289	CD1	LEU	533	7.283	70.472	8.038	1.00	14.73	C
ATOM	290	CD2	LEU	533	9.060	72.183	8.282	1.00	8.85	C
ATOM	291	N	LYS	534	10.899	67.640	8.704	1.00	12.22	N
ATOM	292	CA	LYS	534	11.766	66.936	7.785	1.00	13.88	C
ATOM	293	C	LYS	534	11.337	65.492	7.592	1.00	13.67	C
ATOM	294	O	LYS	534	11.356	64.974	6.478	1.00	19.68	O
ATOM	295	CB	LYS	534	13.210	67.016	8.282	1.00	17.43	C
ATOM	296	CG	LYS	534	13.819	68.393	8.100	1.00	13.24	C
ATOM	297	CD	LYS	534	15.002	68.635	9.036	1.00	18.41	C
ATOM	298	CE	LYS	534	15.936	67.455	9.114	1.00	20.81	C
ATOM	299	NZ	LYS	534	17.017	67.696	10.101	1.00	18.52	N

TABLE 8-continued

Atomic Coordinates for Residues of a Crystal of murine VWF-A1 (SEQ ID NO: 10).

ATOM	300	N	ALA	535	10.871	64.858	8.656	1.00	13.06	N
ATOM	301	CA	ALA	535	10.442	63.472	8.563	1.00	9.85	C
ATOM	302	C	ALA	535	9.164	63.382	7.745	1.00	13.30	C
ATOM	303	O	ALA	535	8.911	62.361	7.082	1.00	15.28	O
ATOM	304	CB	ALA	535	10.254	62.901	9.923	1.00	8.53	C
ATOM	305	N	PHE	536	8.348	64.428	7.797	1.00	13.50	N
ATOM	306	CA	PHE	536	7.117	64.490	6.996	1.00	11.73	C
ATOM	307	C	PHE	536	7.537	64.558	5.521	1.00	14.75	C
ATOM	308	O	PHE	536	6.962	63.887	4.654	1.00	17.83	O
ATOM	309	CB	PHE	536	6.369	65.756	7.350	1.00	6.75	C
ATOM	310	CG	PHE	536	5.252	66.076	6.419	1.00	12.43	C
ATOM	311	CD1	PHE	536	4.113	65.288	6.388	1.00	13.94	C
ATOM	312	CD2	PHE	536	5.314	67.191	5.585	1.00	10.83	C
ATOM	313	CE1	PHE	536	3.040	65.604	5.534	1.00	15.05	C
ATOM	314	CE2	PHE	536	4.260	67.511	4.736	1.00	11.68	C
ATOM	315	CZ	PHE	536	3.114	66.720	4.711	1.00	9.74	C
ATOM	316	N	VAL	537	8.550	65.375	5.250	1.00	13.66	N
ATOM	317	CA	VAL	537	9.068	65.549	3.903	1.00	14.58	C
ATOM	318	C	VAL	537	9.593	64.209	3.418	1.00	16.40	C
ATOM	319	O	VAL	537	9.176	63.735	2.376	1.00	21.04	O
ATOM	320	CB	VAL	537	10.143	66.684	3.858	1.00	12.13	C
ATOM	321	CG1	VAL	537	10.864	66.706	2.527	1.00	7.80	C
ATOM	322	CG2	VAL	537	9.459	68.040	4.070	1.00	2.11	C
ATOM	323	N	VAL	538	10.397	63.541	4.231	1.00	17.90	N
ATOM	324	CA	VAL	538	10.929	62.228	3.867	1.00	15.91	C
ATOM	325	C	VAL	538	9.842	61.187	3.559	1.00	17.55	C
ATOM	326	O	VAL	538	9.946	60.491	2.538	1.00	15.35	O
ATOM	327	CB	VAL	538	11.893	61.693	4.948	1.00	17.65	C
ATOM	328	CG1	VAL	538	12.129	60.169	4.791	1.00	9.43	C
ATOM	329	CG2	VAL	538	13.200	62.447	4.849	1.00	9.34	C
ATOM	330	N	ASP	539	8.818	61.040	4.410	1.00	17.17	N
ATOM	331	CA	ASP	539	7.798	60.050	4.068	1.00	21.22	C
ATOM	332	C	ASP	539	6.892	60.418	2.919	1.00	17.63	C
ATOM	333	O	ASP	539	6.313	59.526	2.305	1.00	20.04	O
ATOM	334	CB	ASP	539	7.048	59.412	5.257	1.00	29.14	C
ATOM	335	CG	ASP	539	6.836	60.345	6.384	1.00	35.47	C
ATOM	336	OD1	ASP	539	6.283	61.428	6.128	1.00	44.75	O
ATOM	337	OD2	ASP	539	7.219	59.993	7.523	1.00	36.63	O
ATOM	338	N	MET	540	6.810	61.709	2.601	1.00	16.39	N
ATOM	339	CA	MET	540	6.063	62.172	1.428	1.00	13.96	C
ATOM	340	C	MET	540	6.857	61.593	0.269	1.00	10.86	C
ATOM	341	O	MET	540	6.333	60.862	−0.539	1.00	14.71	O
ATOM	342	CB	MET	540	6.159	63.688	1.294	1.00	22.58	C
ATOM	343	CG	MET	540	5.149	64.497	2.065	1.00	31.44	C
ATOM	344	SD	MET	540	3.503	64.541	1.326	1.00	45.37	S
ATOM	345	CE	MET	540	3.679	65.729	0.060	1.00	31.22	C
ATOM	346	N	MET	541	8.163	61.870	0.261	1.00	13.18	N
ATOM	347	CA	MET	541	9.116	61.398	−0.751	1.00	10.35	C
ATOM	348	C	MET	541	9.107	59.895	−0.969	1.00	16.49	C
ATOM	349	O	MET	541	9.225	59.438	−2.111	1.00	20.40	O
ATOM	350	CB	MET	541	10.537	61.813	−0.370	1.00	9.58	C
ATOM	351	CG	MET	541	10.820	63.276	−0.620	1.00	11.21	C
ATOM	352	SD	MET	541	12.434	63.842	−0.058	1.00	12.89	S
ATOM	353	CE	MET	541	13.392	63.835	−1.500	1.00	15.28	C
ATOM	354	N	GLU	542	8.985	59.119	0.107	1.00	16.56	N
ATOM	355	CA	GLU	542	8.971	57.657	−0.008	1.00	13.92	C
ATOM	356	C	GLU	542	7.759	57.174	−0.818	1.00	14.26	C
ATOM	357	O	GLU	542	7.789	56.104	−1.430	1.00	15.31	O
ATOM	358	CB	GLU	542	8.925	57.018	1.382	1.00	12.65	C
ATOM	359	CG	GLU	542	10.175	57.228	2.230	1.00	15.01	C
ATOM	360	CD	GLU	542	10.155	56.422	3.528	1.00	13.47	C
ATOM	361	OE1	GLU	542	9.181	55.694	3.785	1.00	18.27	O
ATOM	362	OE2	GLU	542	11.109	56.518	4.308	1.00	15.89	O
ATOM	363	N	ARG	543	6.688	57.961	−0.789	1.00	12.61	N
ATOM	364	CA	ARG	543	5.451	57.633	−1.473	1.00	10.82	C
ATOM	365	C	ARG	543	5.388	58.327	−2.827	1.00	11.37	C
ATOM	366	O	ARG	543	4.310	58.574	−3.373	1.00	13.23	O
ATOM	367	CB	ARG	543	4.285	58.069	−0.593	1.00	9.75	C
ATOM	368	CG	ARG	543	4.168	57.203	0.632	1.00	8.93	C
ATOM	369	CD	ARG	543	3.353	57.841	1.737	1.00	9.66	C
ATOM	370	NE	ARG	543	3.241	56.878	2.835	1.00	17.05	N
ATOM	371	CZ	ARG	543	4.196	56.652	3.737	1.00	12.00	C
ATOM	372	NH1	ARG	543	5.341	57.332	3.697	1.00	9.90	N

TABLE 8-continued

Atomic Coordinates for Residues of a Crystal of murine VWF-A1 (SEQ ID NO: 10).

ATOM	373	NH2	ARG	543	4.044	55.681	4.634	1.00	15.47	N
ATOM	374	N	LEU	544	6.554	58.559	−3.401	1.00	13.34	N
ATOM	375	CA	LEU	544	6.676	59.214	−4.688	1.00	15.24	C
ATOM	376	C	LEU	544	7.606	58.388	−5.574	1.00	16.79	C
ATOM	377	O	LEU	544	8.633	57.871	−5.103	1.00	17.01	O
ATOM	378	CB	LEU	544	7.318	60.578	−4.465	1.00	12.06	C
ATOM	379	CG	LEU	544	6.539	61.886	−4.546	1.00	11.78	C
ATOM	380	CD1	LEU	544	5.138	61.769	−4.046	1.00	11.41	C
ATOM	381	CD2	LEU	544	7.315	62.896	−3.751	1.00	8.97	C
ATOM	382	N	ARG	545	7.283	58.269	−6.855	1.00	15.28	N
ATOM	383	CA	ARG	545	8.187	57.551	−7.740	1.00	15.08	C
ATOM	384	C	ARG	545	9.110	58.627	−8.278	1.00	17.33	C
ATOM	385	O	ARG	545	8.794	59.266	−9.283	1.00	19.41	O
ATOM	386	CB	ARG	545	7.431	56.887	−8.875	1.00	16.99	C
ATOM	387	CG	ARG	545	8.327	56.142	−9.837	1.00	20.50	C
ATOM	388	CD	ARG	545	7.453	55.493	−10.868	1.00	26.46	C
ATOM	389	NE	ARG	545	8.195	54.796	−11.903	1.00	28.51	N
ATOM	390	CZ	ARG	545	7.644	53.877	−12.690	1.00	33.26	C
ATOM	391	NH1	ARG	545	6.357	53.572	−12.541	1.00	31.05	N
ATOM	392	NH2	ARG	545	8.368	53.259	−13.617	1.00	30.37	N
ATOM	393	N	ILE	546	10.205	58.879	−7.556	1.00	18.27	N
ATOM	394	CA	ILE	546	11.177	59.909	−7.919	1.00	18.03	C
ATOM	395	C	ILE	546	12.039	59.480	−9.084	1.00	19.30	C
ATOM	396	O	ILE	546	12.750	58.477	−9.014	1.00	20.66	O
ATOM	397	CB	ILE	546	12.065	60.290	−6.714	1.00	13.91	C
ATOM	398	CG1	ILE	546	11.234	60.956	−5.622	1.00	10.37	C
ATOM	399	CG2	ILE	546	13.134	61.243	−7.134	1.00	10.93	C
ATOM	400	CD1	ILE	546	11.981	61.088	−4.306	1.00	8.23	C
ATOM	401	N	SER	547	11.986	60.270	−10.152	1.00	20.13	N
ATOM	402	CA	SER	547	12.725	59.968	−11.360	1.00	18.38	C
ATOM	403	C	SER	547	12.518	61.108	−12.348	1.00	20.11	C
ATOM	404	O	SER	547	11.443	61.710	−12.407	1.00	19.86	O
ATOM	405	CB	SER	547	12.191	58.664	−11.956	1.00	19.67	C
ATOM	406	OG	SER	547	12.939	58.238	−13.078	1.00	24.56	O
ATOM	407	N	GLN	548	13.542	61.364	−13.156	1.00	21.04	N
ATOM	408	CA	GLN	548	13.527	62.426	−14.159	1.00	22.05	C
ATOM	409	C	GLN	548	12.335	62.253	−15.121	1.00	23.36	C
ATOM	410	O	GLN	548	11.802	63.236	−15.645	1.00	26.68	O
ATOM	411	CB	GLN	548	14.869	62.399	−14.905	1.00	23.09	C
ATOM	412	CG	GLN	548	15.601	63.727	−15.086	1.00	25.87	C
ATOM	413	CD	GLN	548	15.738	64.560	−13.825	1.00	27.98	C
ATOM	414	OE1	GLN	548	15.630	65.783	−13.890	1.00	34.12	O
ATOM	415	NE2	GLN	548	15.977	63.925	−12.684	1.00	25.55	N
ATOM	416	N	LYS	549	11.894	61.009	−15.304	1.00	21.82	N
ATOM	417	CA	LYS	549	10.766	60.701	−16.182	1.00	21.44	C
ATOM	418	C	LYS	549	9.453	60.554	−15.390	1.00	22.62	C
ATOM	419	O	LYS	549	8.374	60.330	−15.971	1.00	18.28	O
ATOM	420	CB	LYS	549	11.041	59.406	−16.955	1.00	24.39	C
ATOM	421	CG	LYS	549	12.431	59.320	−17.575	1.00	34.15	C
ATOM	422	CD	LYS	549	12.762	60.573	−18.390	1.00	44.91	C
ATOM	423	CE	LYS	549	14.154	60.513	−19.044	1.00	51.62	C
ATOM	424	NZ	LYS	549	15.302	60.529	−18.076	1.00	53.07	N
ATOM	425	N	TRP	550	9.535	60.665	−14.064	1.00	19.15	N
ATOM	426	CA	TRP	550	8.350	60.544	−13.241	1.00	18.15	C
ATOM	427	C	TRP	550	8.178	61.740	−12.313	1.00	17.14	C
ATOM	428	O	TRP	550	7.755	62.808	−12.759	1.00	17.54	O
ATOM	429	CB	TRP	550	8.383	59.219	−12.473	1.00	22.31	C
ATOM	430	CG	TRP	550	8.360	58.034	−13.381	1.00	24.07	C
ATOM	431	CD1	TRP	550	9.420	57.250	−13.752	1.00	25.74	C
ATOM	432	CD2	TRP	550	7.228	57.533	−14.096	1.00	23.63	C
ATOM	433	NE1	TRP	550	9.016	56.302	−14.667	1.00	25.59	N
ATOM	434	CE2	TRP	550	7.665	56.461	−14.894	1.00	25.02	C
ATOM	435	CE3	TRP	550	5.875	57.905	−14.136	1.00	23.56	C
ATOM	436	CZ2	TRP	550	6.807	55.743	−15.734	1.00	25.72	C
ATOM	437	CZ3	TRP	550	5.017	57.196	−14.970	1.00	25.40	C
ATOM	438	CH2	TRP	550	5.487	56.125	−15.758	1.00	24.34	C
ATOM	439	N	VAL	551	8.465	61.572	−11.022	1.00	18.80	N
ATOM	440	CA	VAL	551	8.321	62.674	−10.069	1.00	16.63	C
ATOM	441	C	VAL	551	9.695	63.240	−9.739	1.00	14.99	C
ATOM	442	O	VAL	551	10.599	62.501	−9.362	1.00	15.74	O
ATOM	443	CB	VAL	551	7.614	62.214	−8.731	1.00	16.18	C
ATOM	444	CG1	VAL	551	7.477	63.406	−7.761	1.00	10.00	C
ATOM	445	CG2	VAL	551	6.231	61.611	−9.016	1.00	12.35	C

TABLE 8-continued

Atomic Coordinates for Residues of a Crystal of murine VWF-A1 (SEQ ID NO: 10).

ATOM	446	N	ARG	552	9.876	64.536	−9.950	1.00	14.51	N
ATOM	447	CA	ARG	552	11.142	65.180	−9.612	1.00	14.95	C
ATOM	448	C	ARG	552	10.860	66.010	−8.358	1.00	14.50	C
ATOM	449	O	ARG	552	9.756	66.543	−8.188	1.00	12.82	O
ATOM	450	CB	ARG	552	11.639	66.066	−10.757	1.00	13.60	C
ATOM	451	CG	ARG	552	11.890	65.318	−12.052	1.00	14.38	C
ATOM	452	CD	ARG	552	12.686	66.146	−13.018	1.00	14.42	C
ATOM	453	NE	ARG	552	11.980	67.368	−13.388	1.00	22.75	N
ATOM	454	CZ	ARG	552	12.544	68.383	−14.035	1.00	19.53	C
ATOM	455	NH1	ARG	552	13.824	68.318	−14.381	1.00	24.67	N
ATOM	456	NH2	ARG	552	11.823	69.436	−14.383	1.00	12.20	N
ATOM	457	N	VAL	553	11.844	66.108	−7.469	1.00	18.50	N
ATOM	458	CA	VAL	553	11.654	66.840	−6.209	1.00	19.75	C
ATOM	459	C	VAL	553	12.740	67.861	−5.972	1.00	19.66	C
ATOM	460	O	VAL	553	13.872	67.695	−6.406	1.00	22.27	O
ATOM	461	CB	VAL	553	11.677	65.886	−4.956	1.00	18.81	C
ATOM	462	CG1	VAL	553	10.441	64.952	−4.920	1.00	7.54	C
ATOM	463	CG2	VAL	553	12.966	65.070	−4.942	1.00	19.00	C
ATOM	464	N	ALA	554	12.385	68.930	−5.279	1.00	17.64	N
ATOM	465	CA	ALA	554	13.356	69.958	−4.924	1.00	16.45	C
ATOM	466	C	ALA	554	13.038	70.208	−3.450	1.00	14.99	C
ATOM	467	O	ALA	554	11.882	70.006	−3.030	1.00	12.08	O
ATOM	468	CB	ALA	554	13.153	71.224	−5.764	1.00	6.96	C
ATOM	469	N	VAL	555	14.063	70.516	−2.651	1.00	13.54	N
ATOM	470	CA	VAL	555	13.879	70.778	−1.220	1.00	14.53	C
ATOM	471	C	VAL	555	14.590	72.068	−0.892	1.00	11.41	C
ATOM	472	O	VAL	555	15.769	72.191	−1.175	1.00	10.57	O
ATOM	473	CB	VAL	555	14.402	69.594	−0.318	1.00	14.50	C
ATOM	474	CG1	VAL	555	14.355	69.980	1.182	1.00	10.37	C
ATOM	475	CG2	VAL	555	13.491	68.358	−0.515	1.00	11.88	C
ATOM	476	N	VAL	556	13.857	73.044	−0.359	1.00	11.91	N
ATOM	477	CA	VAL	556	14.424	74.337	−0.026	1.00	14.48	C
ATOM	478	C	VAL	556	13.996	74.729	1.394	1.00	18.03	C
ATOM	479	O	VAL	556	12.807	74.771	1.717	1.00	18.24	O
ATOM	480	CB	VAL	556	13.929	75.435	−0.999	1.00	16.63	C
ATOM	481	CG1	VAL	556	14.721	76.700	−0.812	1.00	12.12	C
ATOM	482	CG2	VAL	556	14.022	74.948	−2.450	1.00	17.97	C
ATOM	483	N	GLU	557	14.977	75.023	2.228	1.00	15.76	N
ATOM	484	CA	GLU	557	14.750	75.431	3.594	1.00	16.18	C
ATOM	485	C	GLU	557	14.810	76.937	3.456	1.00	19.44	C
ATOM	486	O	GLU	557	15.751	77.448	2.847	1.00	20.78	O
ATOM	487	CB	GLU	557	15.922	74.921	4.451	1.00	21.73	C
ATOM	488	CG	GLU	557	16.042	75.479	5.890	1.00	22.98	C
ATOM	489	CD	GLU	557	17.093	76.581	6.011	1.00	26.66	C
ATOM	490	OE1	GLU	557	18.322	76.271	6.047	1.00	27.04	O
ATOM	491	OE2	GLU	557	16.666	77.755	6.075	1.00	18.13	O
ATOM	492	N	TYR	558	13.823	77.668	3.964	1.00	16.82	N
ATOM	493	CA	TYR	558	13.878	79.117	3.826	1.00	19.30	C
ATOM	494	C	TYR	558	13.894	79.898	5.123	1.00	21.56	C
ATOM	495	O	TYR	558	13.367	79.450	6.146	1.00	18.80	O
ATOM	496	CB	TYR	558	12.769	79.638	2.903	1.00	18.20	C
ATOM	497	CG	TYR	558	11.350	79.457	3.414	1.00	21.51	C
ATOM	498	CD1	TYR	558	10.681	78.250	3.251	1.00	19.46	C
ATOM	499	CD2	TYR	558	10.662	80.511	4.037	1.00	17.80	C
ATOM	500	CE1	TYR	558	9.360	78.101	3.689	1.00	17.32	C
ATOM	501	CE2	TYR	558	9.361	80.361	4.462	1.00	11.71	C
ATOM	502	CZ	TYR	558	8.727	79.158	4.289	1.00	14.15	C
ATOM	503	OH	TYR	558	7.443	78.997	4.727	1.00	21.33	O
ATOM	504	N	HIS	559	14.548	81.054	5.077	1.00	22.82	N
ATOM	505	CA	HIS	559	14.659	81.942	6.228	1.00	22.57	C
ATOM	506	C	HIS	559	14.761	83.343	5.632	1.00	24.28	C
ATOM	507	O	HIS	559	13.803	83.820	5.035	1.00	24.51	O
ATOM	508	CB	HIS	559	15.877	81.580	7.084	1.00	23.05	C
ATOM	509	CG	HIS	559	17.138	81.409	6.295	1.00	31.12	C
ATOM	510	ND1	HIS	559	17.327	80.384	5.400	1.00	35.63	N
ATOM	511	CD2	HIS	559	18.274	82.154	6.254	1.00	35.29	C
ATOM	512	CE1	HIS	559	18.512	80.499	4.841	1.00	34.53	C
ATOM	513	NE2	HIS	559	19.109	81.564	5.342	1.00	33.84	N
ATOM	514	N	ASP	560	15.895	84.013	5.788	1.00	29.85	N
ATOM	515	CA	ASP	560	16.077	85.336	5.194	1.00	37.48	C
ATOM	516	C	ASP	560	16.114	85.132	3.676	1.00	36.28	C
ATOM	517	O	ASP	560	15.522	85.892	2.912	1.00	39.28	O
ATOM	518	CB	ASP	560	17.391	85.957	5.686	1.00	46.15	C

TABLE 8-continued

Atomic Coordinates for Residues of a Crystal of murine VWF-A1 (SEQ ID NO: 10).

ATOM	519	CG	ASP	560	17.242	87.430	6.059	1.00	55.52	C
ATOM	520	OD1	ASP	560	16.086	87.905	6.195	1.00	60.53	O
ATOM	521	OD2	ASP	560	18.286	88.112	6.218	1.00	59.22	O
ATOM	522	N	GLY	561	16.849	84.101	3.271	1.00	34.51	N
ATOM	523	CA	GLY	561	16.973	83.729	1.879	1.00	29.45	C
ATOM	524	C	GLY	561	16.599	82.261	1.769	1.00	28.57	C
ATOM	525	O	GLY	561	15.991	81.698	2.689	1.00	25.19	O
ATOM	526	N	SER	562	17.044	81.614	0.700	1.00	27.35	N
ATOM	527	CA	SER	562	16.730	80.216	0.502	1.00	26.74	C
ATOM	528	C	SER	562	17.984	79.376	0.408	1.00	28.35	C
ATOM	529	O	SER	562	18.981	79.803	−0.164	1.00	29.74	O
ATOM	530	CB	SER	562	15.903	80.028	−0.770	1.00	25.65	C
ATOM	531	OG	SER	562	14.733	80.834	−0.746	1.00	25.69	O
ATOM	532	N	HIS	563	17.952	78.221	1.058	1.00	26.61	N
ATOM	533	CA	HIS	563	19.052	77.273	1.016	1.00	28.05	C
ATOM	534	C	HIS	563	18.452	76.026	0.354	1.00	25.45	C
ATOM	535	O	HIS	563	17.662	75.317	0.977	1.00	26.54	O
ATOM	536	CB	HIS	563	19.565	76.923	2.425	1.00	33.01	C
ATOM	537	CG	HIS	563	20.592	77.879	2.971	1.00	42.56	C
ATOM	538	ND1	HIS	563	21.368	77.586	4.081	1.00	41.44	N
ATOM	539	CD2	HIS	563	20.950	79.129	2.588	1.00	41.45	C
ATOM	540	CE1	HIS	563	22.148	78.615	4.351	1.00	39.61	C
ATOM	541	NE2	HIS	563	21.919	79.566	3.466	1.00	38.40	N
ATOM	542	N	ALA	564	18.736	75.824	−0.929	1.00	22.09	N
ATOM	543	CA	ALA	564	18.220	74.671	−1.653	1.00	20.28	C
ATOM	544	C	ALA	564	19.183	73.513	−1.505	1.00	18.53	C
ATOM	545	O	ALA	564	20.359	73.650	−1.785	1.00	21.05	O
ATOM	546	CB	ALA	564	18.040	75.005	−3.108	1.00	16.92	C
ATOM	547	N	TYR	565	18.675	72.384	−1.028	1.00	18.56	N
ATOM	548	CA	TYR	565	19.463	71.183	−0.840	1.00	15.69	C
ATOM	549	C	TYR	565	19.236	70.245	−2.021	1.00	16.43	C
ATOM	550	O	TYR	565	20.096	69.461	−2.376	1.00	14.38	O
ATOM	551	CB	TYR	565	19.035	70.473	0.434	1.00	18.28	C
ATOM	552	CG	TYR	565	19.373	71.214	1.691	1.00	20.29	C
ATOM	553	CD1	TYR	565	18.609	72.296	2.105	1.00	19.07	C
ATOM	554	CD2	TYR	565	20.450	70.816	2.485	1.00	24.63	C
ATOM	555	CE1	TYR	565	18.899	72.966	3.277	1.00	23.97	C
ATOM	556	CE2	TYR	565	20.752	71.476	3.670	1.00	25.91	C
ATOM	557	CZ	TYR	565	19.971	72.552	4.061	1.00	24.70	C
ATOM	558	OH	TYR	565	20.247	73.212	5.240	1.00	28.07	O
ATOM	559	N	ILE	566	18.050	70.302	−2.599	1.00	16.43	N
ATOM	560	CA	ILE	566	17.720	69.445	−3.721	1.00	16.83	C
ATOM	561	C	ILE	566	17.121	70.236	−4.876	1.00	14.70	C
ATOM	562	O	ILE	566	16.222	71.044	−4.662	1.00	16.54	O
ATOM	563	CB	ILE	566	16.690	68.368	−3.301	1.00	15.97	C
ATOM	564	CG1	ILE	566	17.187	67.570	−2.097	1.00	18.44	C
ATOM	565	CG2	ILE	566	16.392	67.432	−4.446	1.00	16.90	C
ATOM	566	CD1	ILE	566	18.416	66.752	−2.370	1.00	28.29	C
ATOM	567	N	GLY	567	17.667	70.052	−6.075	1.00	14.70	N
ATOM	568	CA	GLY	567	17.123	70.694	−7.261	1.00	14.69	C
ATOM	569	C	GLY	567	16.372	69.660	−8.106	1.00	15.85	C
ATOM	570	O	GLY	567	16.730	68.469	−8.109	1.00	16.56	O
ATOM	571	N	LEU	568	15.392	70.097	−8.892	1.00	17.74	N
ATOM	572	CA	LEU	568	14.622	69.174	−9.722	1.00	16.77	C
ATOM	573	C	LEU	568	15.471	68.292	−10.628	1.00	18.54	C
ATOM	574	O	LEU	568	15.177	67.117	−10.807	1.00	24.33	O
ATOM	575	CB	LEU	568	13.591	69.935	−10.559	1.00	11.30	C
ATOM	576	CG	LEU	568	12.456	70.603	−9.767	1.00	11.42	C
ATOM	577	CD1	LEU	568	11.744	71.638	−10.640	1.00	6.22	C
ATOM	578	CD2	LEU	568	11.508	69.583	−9.227	1.00	3.91	C
ATOM	579	N	LYS	569	16.512	68.864	−11.212	1.00	23.19	N
ATOM	580	CA	LYS	569	17.393	68.123	−12.124	1.00	23.15	C
ATOM	581	C	LYS	569	18.459	67.248	−11.458	1.00	23.97	C
ATOM	582	O	LYS	569	19.272	66.623	−12.149	1.00	21.55	O
ATOM	583	CB	LYS	569	18.087	69.098	−13.067	1.00	23.83	C
ATOM	584	CG	LYS	569	17.154	69.828	−14.027	1.00	29.42	C
ATOM	585	CD	LYS	569	17.882	71.015	−14.638	1.00	32.89	C
ATOM	586	CE	LYS	569	16.994	71.782	−15.588	1.00	36.32	C
ATOM	587	NZ	LYS	569	17.497	71.604	−16.981	1.00	42.82	N
ATOM	588	N	ASP	570	18.477	67.207	−10.128	1.00	24.14	N
ATOM	589	CA	ASP	570	19.476	66.409	−9.435	1.00	26.34	C
ATOM	590	C	ASP	570	19.300	64.950	−9.749	1.00	31.25	C
ATOM	591	O	ASP	570	18.335	64.336	−9.314	1.00	34.28	O

TABLE 8-continued

Atomic Coordinates for Residues of a Crystal of murine VWF-A1 (SEQ ID NO: 10).

ATOM	592	CB	ASP	570	19.389	66.624	−7.930	1.00	23.64	C
ATOM	593	CG	ASP	570	20.081	67.890	−7.493	1.00	24.59	C
ATOM	594	OD1	ASP	570	20.678	68.575	−8.352	1.00	27.05	O
ATOM	595	OD2	ASP	570	20.041	68.200	−6.289	1.00	24.49	O
ATOM	596	N	ARG	571	20.223	64.390	−10.523	1.00	34.80	N
ATOM	597	CA	ARG	571	20.126	62.983	−10.869	1.00	35.48	C
ATOM	598	C	ARG	571	20.829	62.155	−9.817	1.00	30.97	C
ATOM	599	O	ARG	571	22.025	61.861	−9.922	1.00	29.82	O
ATOM	600	CB	ARG	571	20.710	62.721	−12.246	1.00	46.16	C
ATOM	601	CG	ARG	571	20.453	61.315	−12.743	1.00	60.41	C
ATOM	602	CD	ARG	571	21.501	60.929	−13.762	1.00	70.06	C
ATOM	603	NE	ARG	571	21.379	59.540	−14.198	1.00	76.92	N
ATOM	604	CZ	ARG	571	22.061	59.032	−15.218	1.00	82.89	C
ATOM	605	NH1	ARG	571	22.910	59.802	−15.892	1.00	86.30	N
ATOM	606	NH2	ARG	571	21.877	57.770	−15.579	1.00	85.49	N
ATOM	607	N	LYS	572	20.093	61.876	−8.750	1.00	23.93	N
ATOM	608	CA	LYS	572	20.588	61.086	−7.634	1.00	20.81	C
ATOM	609	C	LYS	572	19.511	60.067	−7.365	1.00	16.06	C
ATOM	610	O	LYS	572	18.381	60.237	−7.789	1.00	17.31	O
ATOM	611	CB	LYS	572	20.843	61.966	−6.393	1.00	18.06	C
ATOM	612	CG	LYS	572	22.004	62.929	−6.578	1.00	20.31	C
ATOM	613	CD	LYS	572	22.138	63.949	−5.468	1.00	23.14	C
ATOM	614	CE	LYS	572	23.202	64.988	−5.836	1.00	24.18	C
ATOM	615	NZ	LYS	572	23.545	65.977	−4.755	1.00	20.57	N
ATOM	616	N	ARG	573	19.857	58.956	−6.750	1.00	20.74	N
ATOM	617	CA	ARG	573	18.834	57.949	−6.475	1.00	22.56	C
ATOM	618	C	ARG	573	17.883	58.457	−5.379	1.00	20.74	C
ATOM	619	O	ARG	573	18.280	59.311	−4.565	1.00	23.29	O
ATOM	620	CB	ARG	573	19.498	56.617	−6.138	1.00	27.37	C
ATOM	621	CG	ARG	573	20.682	56.741	−5.228	1.00	35.56	C
ATOM	622	CD	ARG	573	21.369	55.399	−5.040	1.00	44.36	C
ATOM	623	NE	ARG	573	22.340	55.095	−6.087	1.00	49.02	N
ATOM	624	CZ	ARG	573	22.261	54.052	−6.916	1.00	54.28	C
ATOM	625	NH1	ARG	573	21.240	53.191	−6.847	1.00	56.03	N
ATOM	626	NH2	ARG	573	23.226	53.851	−7.805	1.00	52.89	N
ATOM	627	N	PRO	574	16.610	57.997	−5.375	1.00	17.46	N
ATOM	628	CA	PRO	574	15.610	58.412	−4.392	1.00	12.01	C
ATOM	629	C	PRO	574	16.092	58.327	−2.953	1.00	14.44	C
ATOM	630	O	PRO	574	15.816	59.220	−2.163	1.00	17.36	O
ATOM	631	CB	PRO	574	14.472	57.442	−4.650	1.00	16.28	C
ATOM	632	CG	PRO	574	14.547	57.219	−6.088	1.00	11.38	C
ATOM	633	CD	PRO	574	16.024	57.002	−6.291	1.00	18.26	C
ATOM	634	N	SER	575	16.880	57.305	−2.639	1.00	13.89	N
ATOM	635	CA	SER	575	17.394	57.104	−1.289	1.00	15.54	C
ATOM	636	C	SER	575	18.385	58.170	−0.880	1.00	18.01	C
ATOM	637	O	SER	575	18.394	58.619	0.269	1.00	19.91	O
ATOM	638	CB	SER	575	18.052	55.742	−1.163	1.00	11.08	C
ATOM	639	OG	SER	575	19.038	55.601	−2.157	1.00	15.54	O
ATOM	640	N	GLU	576	19.221	58.599	−1.810	1.00	19.82	N
ATOM	641	CA	GLU	576	20.180	59.616	−1.445	1.00	19.22	C
ATOM	642	C	GLU	576	19.456	60.923	−1.244	1.00	19.69	C
ATOM	643	O	GLU	576	19.754	61.678	−0.322	1.00	21.30	O
ATOM	644	CB	GLU	576	21.287	59.774	−2.483	1.00	24.50	C
ATOM	645	CG	GLU	576	22.414	60.647	−1.930	1.00	34.07	C
ATOM	646	CD	GLU	576	23.375	61.108	−2.979	1.00	44.16	C
ATOM	647	OE1	GLU	576	23.681	60.305	−3.893	1.00	48.95	O
ATOM	648	OE2	GLU	576	23.816	62.283	−2.891	1.00	50.40	O
ATOM	649	N	LEU	577	18.495	61.191	−2.106	1.00	18.39	N
ATOM	650	CA	LEU	577	17.734	62.419	−1.993	1.00	18.06	C
ATOM	651	C	LEU	577	17.042	62.469	−0.640	1.00	15.66	C
ATOM	652	O	LEU	577	17.081	63.482	0.056	1.00	16.14	O
ATOM	653	CB	LEU	577	16.715	62.518	−3.141	1.00	14.46	C
ATOM	654	CG	LEU	577	17.362	62.681	−4.522	1.00	13.01	C
ATOM	655	CD1	LEU	577	16.309	62.686	−5.592	1.00	12.16	C
ATOM	656	CD2	LEU	577	18.182	63.963	−4.564	1.00	10.44	C
ATOM	657	N	ARG	578	16.431	61.356	−0.258	1.00	18.03	N
ATOM	658	CA	ARG	578	15.721	61.282	1.013	1.00	16.42	C
ATOM	659	C	ARG	578	16.656	61.533	2.177	1.00	18.59	C
ATOM	660	O	ARG	578	16.276	62.207	3.136	1.00	23.30	O
ATOM	661	CB	ARG	578	15.005	59.939	1.169	1.00	14.49	C
ATOM	662	CG	ARG	578	13.822	59.783	0.220	1.00	9.10	C
ATOM	663	CD	ARG	578	12.924	58.631	0.583	1.00	7.18	C
ATOM	664	NE	ARG	578	13.668	57.391	0.758	1.00	13.10	N

TABLE 8-continued

Atomic Coordinates for Residues of a Crystal of murine VWF-A1 (SEQ ID NO: 10).

ATOM	665	CZ	ARG	578	13.780	56.455	−0.164	1.00	11.87	C
ATOM	666	NH1	ARG	578	13.191	56.622	−1.340	1.00	9.76	N
ATOM	667	NH2	ARG	578	14.501	55.368	0.083	1.00	14.50	N
ATOM	668	N	ARG	579	17.889	61.046	2.070	1.00	17.36	N
ATOM	669	CA	ARG	579	18.879	61.248	3.119	1.00	17.76	C
ATOM	670	C	ARG	579	19.246	62.719	3.237	1.00	18.99	C
ATOM	671	O	ARG	579	19.372	63.252	4.341	1.00	21.54	O
ATOM	672	CB	ARG	579	20.132	60.414	2.833	1.00	18.77	C
ATOM	673	CG	ARG	579	21.347	60.787	3.684	1.00	24.17	C
ATOM	674	CD	ARG	579	22.532	59.857	3.461	1.00	26.24	C
ATOM	675	NE	ARG	579	22.856	59.678	2.045	1.00	36.84	N
ATOM	676	CZ	ARG	579	22.428	58.654	1.296	1.00	39.67	C
ATOM	677	NH1	ARG	579	21.640	57.703	1.800	1.00	37.99	N
ATOM	678	NH2	ARG	579	22.861	58.521	0.052	1.00	40.49	N
ATOM	679	N	ILE	580	19.433	63.377	2.101	1.00	18.54	N
ATOM	680	CA	ILE	580	19.784	64.790	2.098	1.00	14.36	C
ATOM	681	C	ILE	580	18.681	65.556	2.800	1.00	14.39	C
ATOM	682	O	ILE	580	18.956	66.396	3.643	1.00	15.74	O
ATOM	683	CB	ILE	580	19.969	65.315	0.673	1.00	10.89	C
ATOM	684	CG1	ILE	580	21.209	64.682	0.067	1.00	16.10	C
ATOM	685	CG2	ILE	580	20.087	66.835	0.663	1.00	13.34	C
ATOM	686	CD1	ILE	580	21.457	65.030	−1.408	1.00	15.50	C
ATOM	687	N	ALA	581	17.432	65.208	2.508	1.00	16.77	N
ATOM	688	CA	ALA	581	16.294	65.881	3.121	1.00	16.31	C
ATOM	689	C	ALA	581	16.252	65.723	4.646	1.00	17.47	C
ATOM	690	O	ALA	581	15.942	66.670	5.350	1.00	18.94	O
ATOM	691	CB	ALA	581	14.991	65.386	2.487	1.00	12.38	C
ATOM	692	N	SER	582	16.559	64.539	5.167	1.00	21.47	N
ATOM	693	CA	SER	582	16.516	64.344	6.617	1.00	22.46	C
ATOM	694	C	SER	582	17.700	64.984	7.295	1.00	23.44	C
ATOM	695	O	SER	582	17.673	65.229	8.497	1.00	29.23	O
ATOM	696	CB	SER	582	16.469	62.867	6.974	1.00	21.25	C
ATOM	697	OG	SER	582	17.659	62.228	6.577	1.00	25.04	O
ATOM	698	N	GLN	583	18.728	65.293	6.522	1.00	22.77	N
ATOM	699	CA	GLN	583	19.919	65.910	7.077	1.00	23.36	C
ATOM	700	C	GLN	583	19.874	67.425	6.981	1.00	21.29	C
ATOM	701	O	GLN	583	20.812	68.117	7.416	1.00	20.02	O
ATOM	702	CB	GLN	583	21.177	65.369	6.398	1.00	30.71	C
ATOM	703	CG	GLN	583	21.524	63.939	6.797	1.00	42.85	C
ATOM	704	CD	GLN	583	22.843	63.455	6.192	1.00	55.80	C
ATOM	705	OE1	GLN	583	23.589	62.715	6.839	1.00	62.99	O
ATOM	706	NE2	GLN	583	23.141	63.873	4.950	1.00	59.38	N
ATOM	707	N	VAL	584	18.804	67.940	6.389	1.00	15.51	N
ATOM	708	CA	VAL	584	18.622	69.371	6.255	1.00	21.88	C
ATOM	709	C	VAL	584	18.891	70.018	7.615	1.00	26.42	C
ATOM	710	O	VAL	584	18.367	69.585	8.643	1.00	26.77	O
ATOM	711	CB	VAL	584	17.194	69.706	5.768	1.00	25.45	C
ATOM	712	CG1	VAL	584	16.944	71.199	5.861	1.00	30.17	C
ATOM	713	CG2	VAL	584	17.013	69.269	4.333	1.00	27.33	C
ATOM	714	N	LYS	585	19.690	71.073	7.597	1.00	30.58	N
ATOM	715	CA	LYS	585	20.108	71.788	8.798	1.00	33.89	C
ATOM	716	C	LYS	585	19.091	72.734	9.417	1.00	32.68	C
ATOM	717	O	LYS	585	18.570	73.641	8.747	1.00	33.70	O
ATOM	718	CB	LYS	585	21.398	72.551	8.497	1.00	41.79	C
ATOM	719	CG	LYS	585	22.643	71.977	9.165	1.00	52.09	C
ATOM	720	CD	LYS	585	22.693	72.340	10.657	1.00	63.92	C
ATOM	721	CE	LYS	585	22.857	73.859	10.873	1.00	70.35	C
ATOM	722	NZ	LYS	585	22.735	74.271	12.316	1.00	74.91	N
ATOM	723	N	TYR	586	18.859	72.557	10.718	1.00	30.31	N
ATOM	724	CA	TYR	586	17.928	73.416	11.454	1.00	29.54	C
ATOM	725	C	TYR	586	18.583	74.776	11.564	1.00	29.75	C
ATOM	726	O	TYR	586	19.696	74.885	12.079	1.00	30.67	O
ATOM	727	CB	TYR	586	17.619	72.856	12.861	1.00	27.95	C
ATOM	728	CG	TYR	586	16.772	73.793	13.721	1.00	26.56	C
ATOM	729	CD1	TYR	586	15.389	73.906	13.522	1.00	24.59	C
ATOM	730	CD2	TYR	586	17.361	74.604	14.699	1.00	21.69	C
ATOM	731	CE1	TYR	586	14.616	74.806	14.265	1.00	20.84	C
ATOM	732	CE2	TYR	586	16.590	75.508	15.457	1.00	17.53	C
ATOM	733	CZ	TYR	586	15.222	75.601	15.233	1.00	23.50	C
ATOM	734	OH	TYR	586	14.461	76.482	15.978	1.00	21.84	O
ATOM	735	N	ALA	587	17.932	75.788	10.994	1.00	28.03	N
ATOM	736	CA	ALA	587	18.433	77.150	11.016	1.00	27.73	C
ATOM	737	C	ALA	587	17.945	77.932	12.220	1.00	27.05	C

TABLE 8-continued

Atomic Coordinates for Residues of a Crystal of murine VWF-A1 (SEQ ID NO: 10).

ATOM	738	O	ALA	587	18.735	78.601	12.870	1.00	26.39	O
ATOM	739	CB	ALA	587	18.056	77.882	9.719	1.00	29.03	C
ATOM	740	N	GLY	588	16.643	77.880	12.490	1.00	25.16	N
ATOM	741	CA	GLY	588	16.093	78.606	13.619	1.00	24.35	C
ATOM	742	C	GLY	588	16.224	80.115	13.484	1.00	26.00	C
ATOM	743	O	GLY	588	16.582	80.806	14.429	1.00	27.67	O
ATOM	744	N	SER	589	15.891	80.626	12.309	1.00	25.60	N
ATOM	745	CA	SER	589	15.985	82.044	12.009	1.00	25.91	C
ATOM	746	C	SER	589	14.916	82.905	12.679	1.00	27.91	C
ATOM	747	O	SER	589	13.849	82.426	13.050	1.00	23.56	O
ATOM	748	CB	SER	589	15.879	82.228	10.495	1.00	26.02	C
ATOM	749	OG	SER	589	16.935	83.028	9.992	1.00	41.28	O
ATOM	750	N	GLN	590	15.212	84.193	12.811	1.00	32.43	N
ATOM	751	CA	GLN	590	14.256	85.130	13.373	1.00	37.48	C
ATOM	752	C	GLN	590	13.253	85.476	12.296	1.00	35.55	C
ATOM	753	O	GLN	590	12.160	85.937	12.588	1.00	38.88	O
ATOM	754	CB	GLN	590	14.955	86.415	13.821	1.00	39.80	C
ATOM	755	CG	GLN	590	15.288	86.435	15.295	1.00	56.15	C
ATOM	756	CD	GLN	590	16.155	87.622	15.686	1.00	65.66	C
ATOM	757	OE1	GLN	590	15.649	88.723	15.932	1.00	71.38	O
ATOM	758	NE2	GLN	590	17.473	87.403	15.742	1.00	67.58	N
ATOM	759	N	VAL	591	13.647	85.246	11.047	1.00	34.29	N
ATOM	760	CA	VAL	591	12.832	85.570	9.880	1.00	30.91	C
ATOM	761	C	VAL	591	12.827	84.471	8.809	1.00	26.09	C
ATOM	762	O	VAL	591	13.885	84.022	8.392	1.00	23.24	O
ATOM	763	CB	VAL	591	13.359	86.907	9.243	1.00	30.16	C
ATOM	764	CG1	VAL	591	13.067	86.983	7.758	1.00	33.78	C
ATOM	765	CG2	VAL	591	12.750	88.102	9.949	1.00	29.76	C
ATOM	766	N	ALA	592	11.636	84.000	8.437	1.00	24.70	N
ATOM	767	CA	ALA	592	11.462	83.007	7.372	1.00	20.42	C
ATOM	768	C	ALA	592	10.529	83.712	6.375	1.00	23.74	C
ATOM	769	O	ALA	592	9.320	83.852	6.629	1.00	19.55	O
ATOM	770	CB	ALA	592	10.829	81.750	7.893	1.00	14.37	C
ATOM	771	N	SER	593	11.104	84.209	5.277	1.00	22.94	N
ATOM	772	CA	SER	593	10.349	84.951	4.270	1.00	23.81	C
ATOM	773	C	SER	593	9.677	84.093	3.224	1.00	22.86	C
ATOM	774	O	SER	593	10.336	83.470	2.401	1.00	22.82	O
ATOM	775	CB	SER	593	11.232	85.974	3.569	1.00	23.57	C
ATOM	776	OG	SER	593	10.496	86.636	2.556	1.00	14.43	O
ATOM	777	N	THR	594	8.354	84.150	3.212	1.00	22.76	N
ATOM	778	CA	THR	594	7.568	83.380	2.263	1.00	22.25	C
ATOM	779	C	THR	594	7.608	84.069	0.898	1.00	21.75	C
ATOM	780	O	THR	594	7.593	83.411	−0.143	1.00	21.45	O
ATOM	781	CB	THR	594	6.122	83.217	2.768	1.00	19.99	C
ATOM	782	OG1	THR	594	5.593	84.505	3.106	1.00	18.50	O
ATOM	783	CG2	THR	594	6.110	82.375	4.021	1.00	15.10	C
ATOM	784	N	SER	595	7.663	85.400	0.901	1.00	21.20	N
ATOM	785	CA	SER	595	7.738	86.164	−0.334	1.00	18.60	C
ATOM	786	C	SER	595	9.032	85.850	−1.040	1.00	19.69	C
ATOM	787	O	SER	595	9.044	85.513	−2.228	1.00	22.57	O
ATOM	788	CB	SER	595	7.661	87.653	−0.037	1.00	11.10	C
ATOM	789	OG	SER	595	6.344	87.985	0.326	1.00	19.37	O
ATOM	790	N	GLU	596	10.122	85.922	−0.294	1.00	21.05	N
ATOM	791	CA	GLU	596	11.418	85.638	−0.863	1.00	23.66	C
ATOM	792	C	GLU	596	11.591	84.184	−1.299	1.00	22.27	C
ATOM	793	O	GLU	596	12.302	83.905	−2.260	1.00	24.61	O
ATOM	794	CB	GLU	596	12.543	86.151	0.043	1.00	30.29	C
ATOM	795	CG	GLU	596	13.058	87.575	−0.366	1.00	39.81	C
ATOM	796	CD	GLU	596	11.948	88.663	−0.449	1.00	45.46	C
ATOM	797	OE1	GLU	596	11.391	89.067	0.602	1.00	52.14	O
ATOM	798	OE2	GLU	596	11.648	89.142	−1.565	1.00	47.05	O
ATOM	799	N	VAL	597	10.886	83.257	−0.661	1.00	19.54	N
ATOM	800	CA	VAL	597	10.993	81.869	−1.079	1.00	14.34	C
ATOM	801	C	VAL	597	10.056	81.566	−2.257	1.00	14.34	C
ATOM	802	O	VAL	597	10.322	80.677	−3.047	1.00	15.50	O
ATOM	803	CB	VAL	597	10.768	80.893	0.077	1.00	10.95	C
ATOM	804	CG1	VAL	597	9.297	80.603	0.266	1.00	9.01	C
ATOM	805	CG2	VAL	597	11.555	79.624	−0.172	1.00	10.66	C
ATOM	806	N	LEU	598	8.951	82.287	−2.372	1.00	12.70	N
ATOM	807	CA	LEU	598	8.067	82.083	−3.502	1.00	12.15	C
ATOM	808	C	LEU	598	8.749	82.749	−4.699	1.00	17.31	C
ATOM	809	O	LEU	598	8.579	82.324	−5.840	1.00	18.32	O
ATOM	810	CB	LEU	598	6.700	82.699	−3.240	1.00	8.81	C

TABLE 8-continued

Atomic Coordinates for Residues of a Crystal of murine VWF-A1 (SEQ ID NO: 10).

ATOM	811	CG	LEU	598	5.728	81.891	−2.363	1.00	9.60	C
ATOM	812	CD1	LEU	598	4.467	82.716	−2.154	1.00	9.06	C
ATOM	813	CD2	LEU	598	5.370	80.522	−2.975	1.00	7.06	C
ATOM	814	N	LYS	599	9.552	83.777	−4.432	1.00	18.09	N
ATOM	815	CA	LYS	599	10.288	84.442	−5.492	1.00	20.39	C
ATOM	816	C	LYS	599	11.328	83.475	−6.055	1.00	20.58	C
ATOM	817	O	LYS	599	11.552	83.432	−7.260	1.00	23.57	O
ATOM	818	CB	LYS	599	11.001	85.693	−4.982	1.00	21.30	C
ATOM	819	CG	LYS	599	11.836	86.366	−6.063	1.00	20.62	C
ATOM	820	CD	LYS	599	12.331	87.732	−5.633	1.00	28.26	C
ATOM	821	CE	LYS	599	13.674	87.677	−4.914	1.00	28.15	C
ATOM	822	NZ	LYS	599	14.463	88.927	−5.268	1.00	36.90	N
ATOM	823	N	TYR	600	11.972	82.712	−5.184	1.00	15.33	N
ATOM	824	CA	TYR	600	12.975	81.739	−5.605	1.00	14.72	C
ATOM	825	C	TYR	600	12.380	80.614	−6.470	1.00	15.26	C
ATOM	826	O	TYR	600	13.031	80.110	−7.394	1.00	14.23	O
ATOM	827	CB	TYR	600	13.659	81.159	−4.369	1.00	10.22	C
ATOM	828	CG	TYR	600	14.763	80.168	−4.658	1.00	13.36	C
ATOM	829	CD1	TYR	600	16.091	80.594	−4.820	1.00	9.42	C
ATOM	830	CD2	TYR	600	14.488	78.791	−4.745	1.00	9.40	C
ATOM	831	CE1	TYR	600	17.123	79.670	−5.066	1.00	8.00	C
ATOM	832	CE2	TYR	600	15.502	77.874	−4.987	1.00	11.12	C
ATOM	833	CZ	TYR	600	16.812	78.320	−5.154	1.00	13.46	C
ATOM	834	OH	TYR	600	17.796	77.412	−5.477	1.00	18.52	O
ATOM	835	N	THR	601	11.144	80.228	−6.167	1.00	15.74	N
ATOM	836	CA	THR	601	10.449	79.163	−6.887	1.00	16.26	C
ATOM	837	C	THR	601	10.062	79.658	−8.291	1.00	18.06	C
ATOM	838	O	THR	601	10.189	78.942	−9.297	1.00	20.51	O
ATOM	839	CB	THR	601	9.184	78.724	−6.074	1.00	13.62	C
ATOM	840	OG1	THR	601	9.583	78.361	−4.745	1.00	14.88	O
ATOM	841	CG2	THR	601	8.493	77.517	−6.690	1.00	8.81	C
ATOM	842	N	LEU	602	9.660	80.914	−8.358	1.00	18.80	N
ATOM	843	CA	LEU	602	9.239	81.517	−9.594	1.00	12.28	C
ATOM	844	C	LEU	602	10.394	81.808	−10.528	1.00	12.80	C
ATOM	845	O	LEU	602	10.365	81.445	−11.686	1.00	15.88	O
ATOM	846	CB	LEU	602	8.497	82.800	−9.259	1.00	7.41	C
ATOM	847	CG	LEU	602	7.971	83.645	−10.410	1.00	4.81	C
ATOM	848	CD1	LEU	602	7.137	82.788	−11.305	1.00	11.16	C
ATOM	849	CD2	LEU	602	7.163	84.783	−9.881	1.00	3.73	C
ATOM	850	N	PHE	603	11.409	82.496	−10.031	1.00	17.52	N
ATOM	851	CA	PHE	603	12.537	82.883	−10.862	1.00	18.59	C
ATOM	852	C	PHE	603	13.743	81.966	−10.844	1.00	21.46	C
ATOM	853	O	PHE	603	14.577	82.022	−11.746	1.00	21.41	O
ATOM	854	CB	PHE	603	12.954	84.322	−10.533	1.00	13.06	C
ATOM	855	CG	PHE	603	11.860	85.333	−10.758	1.00	13.57	C
ATOM	856	CD1	PHE	603	11.257	85.469	−12.006	1.00	13.30	C
ATOM	857	CD2	PHE	603	11.422	86.138	−9.722	1.00	11.61	C
ATOM	858	CE1	PHE	603	10.232	86.385	−12.212	1.00	8.39	C
ATOM	859	CE2	PHE	603	10.403	87.054	−9.916	1.00	14.44	C
ATOM	860	CZ	PHE	603	9.804	87.180	−11.160	1.00	14.78	C
ATOM	861	N	GLN	604	13.828	81.087	−9.856	1.00	24.28	N
ATOM	862	CA	GLN	604	14.983	80.205	−9.787	1.00	27.12	C
ATOM	863	C	GLN	604	14.687	78.739	−10.058	1.00	24.50	C
ATOM	864	O	GLN	604	15.512	78.040	−10.605	1.00	24.04	O
ATOM	865	CB	GLN	604	15.689	80.368	−8.440	1.00	31.32	C
ATOM	866	CG	GLN	604	16.522	81.621	−8.287	1.00	35.80	C
ATOM	867	CD	GLN	604	17.861	81.469	−8.960	1.00	45.50	C
ATOM	868	OE1	GLN	604	18.784	80.859	−8.418	1.00	49.26	O
ATOM	869	NE2	GLN	604	17.973	81.999	−10.166	1.00	54.22	N
ATOM	870	N	ILE	605	13.521	78.260	−9.660	1.00	23.49	N
ATOM	871	CA	ILE	605	13.196	76.859	−9.881	1.00	22.06	C
ATOM	872	C	ILE	605	12.418	76.627	−11.163	1.00	25.79	C
ATOM	873	O	ILE	605	12.635	75.633	−11.861	1.00	28.80	O
ATOM	874	CB	ILE	605	12.425	76.243	−8.678	1.00	18.12	C
ATOM	875	CG1	ILE	605	13.356	76.119	−7.470	1.00	16.09	C
ATOM	876	CG2	ILE	605	11.862	74.874	−9.035	1.00	15.46	C
ATOM	877	CD1	ILE	605	12.661	75.604	−6.225	1.00	12.12	C
ATOM	878	N	PHE	606	11.444	77.484	−11.431	1.00	21.94	N
ATOM	879	CA	PHE	606	10.670	77.333	−12.645	1.00	22.03	C
ATOM	880	C	PHE	606	10.903	78.556	−13.533	1.00	22.92	C
ATOM	881	O	PHE	606	9.965	79.130	−14.083	1.00	19.90	O
ATOM	882	CB	PHE	606	9.188	77.149	−12.310	1.00	19.38	C
ATOM	883	CG	PHE	606	8.899	75.943	−11.442	1.00	24.03	C

TABLE 8-continued

Atomic Coordinates for Residues of a Crystal of murine VWF-A1 (SEQ ID NO: 10).

ATOM	884	CD1	PHE	606	8.941	74.656	−11.969	1.00	21.11	C
ATOM	885	CD2	PHE	606	8.498	76.107	−10.108	1.00	23.28	C
ATOM	886	CE1	PHE	606	8.582	73.560	−11.178	1.00	22.09	C
ATOM	887	CE2	PHE	606	8.141	75.025	−9.319	1.00	19.77	C
ATOM	888	CZ	PHE	606	8.175	73.749	−9.850	1.00	22.92	C
ATOM	889	N	SER	607	12.165	78.971	−13.641	1.00	27.16	N
ATOM	890	CA	SER	607	12.505	80.127	−14.456	1.00	34.01	C
ATOM	891	C	SER	607	11.927	79.863	−15.832	1.00	41.74	C
ATOM	892	O	SER	607	11.276	80.737	−16.421	1.00	49.40	O
ATOM	893	CB	SER	607	13.997	80.342	−14.543	1.00	29.88	C
ATOM	894	OG	SER	607	14.209	81.653	−15.023	1.00	31.36	O
ATOM	895	N	LYS	608	12.279	78.717	−16.404	1.00	43.35	N
ATOM	896	CA	LYS	608	11.647	78.316	−17.648	1.00	46.17	C
ATOM	897	C	LYS	608	11.480	76.817	−17.716	1.00	42.64	C
ATOM	898	O	LYS	608	12.365	76.029	−17.390	1.00	40.78	O
ATOM	899	CB	LYS	608	12.195	78.939	−18.935	1.00	54.48	C
ATOM	900	CG	LYS	608	11.035	79.221	−19.979	1.00	55.51	C
ATOM	901	CD	LYS	608	9.997	80.303	−19.504	1.00	54.84	C
ATOM	902	CE	LYS	608	8.628	79.732	−19.088	1.00	53.13	C
ATOM	903	NZ	LYS	608	7.777	79.171	−20.196	1.00	50.29	N
ATOM	904	N	ILE	609	10.244	76.475	−18.018	1.00	38.42	N
ATOM	905	CA	ILE	609	9.753	75.134	−18.077	1.00	32.87	C
ATOM	906	C	ILE	609	10.437	74.185	−19.038	1.00	30.65	C
ATOM	907	O	ILE	609	10.108	74.152	−20.208	1.00	34.42	O
ATOM	908	CB	ILE	609	8.235	75.198	−18.302	1.00	31.16	C
ATOM	909	CG1	ILE	609	7.626	76.217	−17.324	1.00	30.77	C
ATOM	910	CG2	ILE	609	7.595	73.836	−18.148	1.00	32.93	C
ATOM	911	CD1	ILE	609	8.177	76.152	−15.893	1.00	27.01	C
ATOM	912	N	ASP	610	11.382	73.407	−18.512	1.00	29.30	N
ATOM	913	CA	ASP	610	12.115	72.397	−19.278	1.00	26.65	C
ATOM	914	C	ASP	610	11.296	71.090	−19.348	1.00	24.52	C
ATOM	915	O	ASP	610	11.667	70.160	−20.061	1.00	22.38	O
ATOM	916	CB	ASP	610	13.472	72.100	−18.610	1.00	31.38	C
ATOM	917	CG	ASP	610	13.326	71.445	−17.208	1.00	40.16	C
ATOM	918	OD1	ASP	610	12.777	72.111	−16.296	1.00	44.05	O
ATOM	919	OD2	ASP	610	13.781	70.280	−17.006	1.00	37.91	O
ATOM	920	N	ARG	611	10.207	71.023	−18.586	1.00	20.53	N
ATOM	921	CA	ARG	611	9.338	69.847	−18.523	1.00	17.10	C
ATOM	922	C	ARG	611	7.882	70.318	−18.565	1.00	18.14	C
ATOM	923	O	ARG	611	7.147	70.151	−17.579	1.00	17.77	O
ATOM	924	CB	ARG	611	9.555	69.117	−17.188	1.00	15.35	C
ATOM	925	CG	ARG	611	10.090	67.715	−17.279	1.00	19.43	C
ATOM	926	CD	ARG	611	9.053	66.659	−16.878	1.00	17.95	C
ATOM	927	NE	ARG	611	9.225	66.220	−15.507	1.00	15.53	N
ATOM	928	CZ	ARG	611	8.787	65.070	−15.009	1.00	15.73	C
ATOM	929	NH1	ARG	611	8.143	64.187	−15.746	1.00	14.15	N
ATOM	930	NH2	ARG	611	8.950	64.830	−13.726	1.00	20.70	N
ATOM	931	N	PRO	612	7.410	70.799	−19.734	1.00	18.33	N
ATOM	932	CA	PRO	612	6.023	71.278	−19.846	1.00	18.14	C
ATOM	933	C	PRO	612	4.985	70.176	−19.746	1.00	16.54	C
ATOM	934	O	PRO	612	3.800	70.458	−19.694	1.00	18.83	O
ATOM	935	CB	PRO	612	6.003	71.928	−21.226	1.00	20.27	C
ATOM	936	CG	PRO	612	6.909	70.998	−22.015	1.00	21.20	C
ATOM	937	CD	PRO	612	8.066	70.757	−21.054	1.00	19.55	C
ATOM	938	N	GLU	613	5.439	68.926	−19.694	1.00	17.56	N
ATOM	939	CA	GLU	613	4.539	67.780	−19.590	1.00	18.04	C
ATOM	940	C	GLU	613	4.265	67.341	−18.137	1.00	20.09	C
ATOM	941	O	GLU	613	3.525	66.376	−17.895	1.00	19.26	O
ATOM	942	CB	GLU	613	5.088	66.593	−20.380	1.00	17.32	C
ATOM	943	CG	GLU	613	6.320	65.939	−19.764	1.00	16.50	C
ATOM	944	CD	GLU	613	7.622	66.575	−20.203	1.00	17.75	C
ATOM	945	OE1	GLU	613	7.609	67.682	−20.775	1.00	21.13	O
ATOM	946	OE2	GLU	613	8.680	65.949	−19.979	1.00	18.68	O
ATOM	947	N	ALA	614	4.896	67.997	−17.172	1.00	17.80	N
ATOM	948	CA	ALA	614	4.645	67.643	−15.782	1.00	17.35	C
ATOM	949	C	ALA	614	3.998	68.805	−15.068	1.00	17.82	C
ATOM	950	O	ALA	614	4.245	69.962	−15.422	1.00	18.03	O
ATOM	951	CB	ALA	614	5.929	67.302	−15.103	1.00	14.29	C
ATOM	952	N	SER	615	3.143	68.502	−14.088	1.00	14.90	N
ATOM	953	CA	SER	615	2.532	69.553	−13.297	1.00	13.08	C
ATOM	954	C	SER	615	3.622	70.036	−12.347	1.00	14.27	C
ATOM	955	O	SER	615	4.577	69.304	−12.056	1.00	17.25	O
ATOM	956	CB	SER	615	1.362	69.029	−12.491	1.00	10.01	C

TABLE 8-continued

Atomic Coordinates for Residues of a Crystal of murine VWF-A1 (SEQ ID NO: 10).

ATOM	957	OG	SER	615	0.379	68.502	−13.348	1.00	13.32	O
ATOM	958	N	ARG	616	3.505	71.290	−11.929	1.00	13.79	N
ATOM	959	CA	ARG	616	4.441	71.923	−11.021	1.00	10.29	C
ATOM	960	C	ARG	616	3.696	72.218	−9.722	1.00	8.30	C
ATOM	961	O	ARG	616	2.638	72.841	−9.721	1.00	8.31	O
ATOM	962	CB	ARG	616	4.966	73.188	−11.671	1.00	10.60	C
ATOM	963	CG	ARG	616	5.580	72.883	−13.020	1.00	11.29	C
ATOM	964	CD	ARG	616	5.648	74.101	−13.902	1.00	18.79	C
ATOM	965	NE	ARG	616	5.380	73.740	−15.286	1.00	25.65	N
ATOM	966	CZ	ARG	616	4.217	73.955	−15.877	1.00	28.89	C
ATOM	967	NH1	ARG	616	3.238	74.543	−15.223	1.00	36.05	N
ATOM	968	NH2	ARG	616	3.977	73.463	−17.075	1.00	37.22	N
ATOM	969	N	ILE	617	4.187	71.673	−8.616	1.00	10.46	N
ATOM	970	CA	ILE	617	3.532	71.893	−7.341	1.00	8.09	C
ATOM	971	C	ILE	617	4.549	72.311	−6.289	1.00	9.44	C
ATOM	972	O	ILE	617	5.625	71.717	−6.176	1.00	13.23	O
ATOM	973	CB	ILE	617	2.790	70.616	−6.898	1.00	13.19	C
ATOM	974	CG1	ILE	617	1.747	70.212	−7.951	1.00	12.54	C
ATOM	975	CG2	ILE	617	2.102	70.832	−5.549	1.00	17.10	C
ATOM	976	CD1	ILE	617	0.818	69.049	−7.489	1.00	9.40	C
ATOM	977	N	ALA	618	4.241	73.396	−5.587	1.00	11.26	N
ATOM	978	CA	ALA	618	5.092	73.932	−4.523	1.00	9.88	C
ATOM	979	C	ALA	618	4.319	73.708	−3.221	1.00	13.13	C
ATOM	980	O	ALA	618	3.164	74.135	−3.084	1.00	13.87	O
ATOM	981	CB	ALA	618	5.332	75.403	−4.747	1.00	6.93	C
ATOM	982	N	LEU	619	4.920	72.964	−2.304	1.00	11.48	N
ATOM	983	CA	LEU	619	4.286	72.655	−1.029	1.00	14.03	C
ATOM	984	C	LEU	619	4.900	73.622	−0.022	1.00	12.75	C
ATOM	985	O	LEU	619	6.035	73.447	0.395	1.00	9.90	O
ATOM	986	CB	LEU	619	4.604	71.206	−0.675	1.00	14.36	C
ATOM	987	CG	LEU	619	3.800	70.416	0.353	1.00	19.68	C
ATOM	988	CD1	LEU	619	2.311	70.465	0.078	1.00	19.00	C
ATOM	989	CD2	LEU	619	4.319	68.989	0.289	1.00	14.02	C
ATOM	990	N	LEU	620	4.186	74.700	0.281	1.00	11.45	N
ATOM	991	CA	LEU	620	4.706	75.711	1.201	1.00	14.00	C
ATOM	992	C	LEU	620	4.397	75.384	2.663	1.00	13.84	C
ATOM	993	O	LEU	620	3.271	75.581	3.123	1.00	10.40	O
ATOM	994	CB	LEU	620	4.156	77.095	0.823	1.00	11.10	C
ATOM	995	CG	LEU	620	4.779	78.300	1.525	1.00	10.11	C
ATOM	996	CD1	LEU	620	6.276	78.366	1.250	1.00	10.19	C
ATOM	997	CD2	LEU	620	4.089	79.568	1.058	1.00	11.71	C
ATOM	998	N	LEU	621	5.397	74.880	3.380	1.00	12.67	N
ATOM	999	CA	LEU	621	5.226	74.528	4.785	1.00	15.42	C
ATOM	1000	C	LEU	621	5.536	75.770	5.639	1.00	15.14	C
ATOM	1001	O	LEU	621	6.697	76.117	5.882	1.00	17.05	O
ATOM	1002	CB	LEU	621	6.117	73.327	5.100	1.00	11.95	C
ATOM	1003	CG	LEU	621	5.761	72.185	4.115	1.00	11.75	C
ATOM	1004	CD1	LEU	621	6.957	71.338	3.727	1.00	7.16	C
ATOM	1005	CD2	LEU	621	4.678	71.351	4.702	1.00	7.13	C
ATOM	1006	N	MET	622	4.467	76.390	6.131	1.00	13.49	N
ATOM	1007	CA	MET	622	4.538	77.624	6.899	1.00	17.11	C
ATOM	1008	C	MET	622	4.273	77.505	8.396	1.00	17.35	C
ATOM	1009	O	MET	622	3.211	77.062	8.803	1.00	18.55	O
ATOM	1010	CB	MET	622	3.499	78.593	6.341	1.00	19.32	C
ATOM	1011	CG	MET	622	3.634	78.898	4.878	1.00	19.64	C
ATOM	1012	SD	MET	622	2.174	79.715	4.255	1.00	22.75	S
ATOM	1013	CE	MET	622	2.302	81.305	4.988	1.00	20.32	C
ATOM	1014	N	ALA	623	5.199	78.003	9.204	1.00	16.59	N
ATOM	1015	CA	ALA	623	5.057	78.000	10.654	1.00	14.97	C
ATOM	1016	C	ALA	623	5.115	79.425	11.201	1.00	18.32	C
ATOM	1017	O	ALA	623	4.952	79.648	12.405	1.00	21.62	O
ATOM	1018	CB	ALA	623	6.167	77.173	11.279	1.00	15.96	C
ATOM	1019	N	SER	624	5.300	80.397	10.323	1.00	19.46	N
ATOM	1020	CA	SER	624	5.433	81.772	10.771	1.00	21.55	C
ATOM	1021	C	SER	624	4.869	82.812	9.799	1.00	22.83	C
ATOM	1022	O	SER	624	4.108	82.483	8.855	1.00	18.76	O
ATOM	1023	CB	SER	624	6.921	82.065	11.026	1.00	18.76	C
ATOM	1024	OG	SER	624	7.721	81.753	9.896	1.00	22.42	O
ATOM	1025	N	GLN	625	5.152	84.075	10.122	1.00	22.23	N
ATOM	1026	CA	GLN	625	4.776	85.204	9.282	1.00	27.39	C
ATOM	1027	C	GLN	625	5.948	86.161	9.222	1.00	27.09	C
ATOM	1028	O	GLN	625	6.583	86.445	10.227	1.00	27.21	O
ATOM	1029	CB	GLN	625	3.498	85.908	9.742	1.00	27.20	C

TABLE 8-continued

Atomic Coordinates for Residues of a Crystal of murine VWF-A1 (SEQ ID NO: 10).

ATOM	1030	CG	GLN	625	3.294	86.097	11.206	1.00	33.26	C
ATOM	1031	CD	GLN	625	1.881	86.577	11.507	1.00	32.60	C
ATOM	1032	OE1	GLN	625	1.168	85.979	12.305	1.00	41.09	O
ATOM	1033	NE2	GLN	625	1.471	87.647	10.854	1.00	32.88	N
ATOM	1034	N	GLU	626	6.326	86.519	8.004	1.00	28.00	N
ATOM	1035	CA	GLU	626	7.434	87.437	7.771	1.00	28.38	C
ATOM	1036	C	GLU	626	7.013	88.885	8.071	1.00	29.02	C
ATOM	1037	O	GLU	626	5.813	89.221	8.041	1.00	25.62	O
ATOM	1038	CB	GLU	626	7.852	87.355	6.304	1.00	25.11	C
ATOM	1039	CG	GLU	626	6.807	87.963	5.378	1.00	23.18	C
ATOM	1040	CD	GLU	626	7.062	87.693	3.915	1.00	24.81	C
ATOM	1041	OE1	GLU	626	8.171	87.208	3.580	1.00	20.13	O
ATOM	1042	OE2	GLU	626	6.129	87.946	3.116	1.00	17.26	O
ATOM	1043	N	PRO	627	7.995	89.762	8.367	1.00	29.70	N
ATOM	1044	CA	PRO	627	7.708	91.173	8.653	1.00	31.82	C
ATOM	1045	C	PRO	627	7.050	91.835	7.441	1.00	33.52	C
ATOM	1046	O	PRO	627	7.505	91.662	6.310	1.00	33.48	O
ATOM	1047	CB	PRO	627	9.096	91.759	8.947	1.00	28.98	C
ATOM	1048	CG	PRO	627	10.037	90.803	8.297	1.00	31.86	C
ATOM	1049	CD	PRO	627	9.420	89.477	8.587	1.00	29.78	C
ATOM	1050	N	GLN	628	6.000	92.609	7.702	1.00	36.73	N
ATOM	1051	CA	GLN	628	5.208	93.302	6.679	1.00	38.04	C
ATOM	1052	C	GLN	628	6.026	93.896	5.542	1.00	35.21	C
ATOM	1053	O	GLN	628	5.629	93.832	4.375	1.00	34.60	O
ATOM	1054	CB	GLN	628	4.353	94.406	7.332	1.00	44.33	C
ATOM	1055	CG	GLN	628	2.922	94.535	6.808	1.00	52.77	C
ATOM	1056	CD	GLN	628	1.885	94.095	7.834	1.00	60.03	C
ATOM	1057	OE1	GLN	628	1.070	94.899	8.302	1.00	61.34	O
ATOM	1058	NE2	GLN	628	1.923	92.814	8.206	1.00	63.65	N
ATOM	1059	N	ARG	629	7.180	94.448	5.884	1.00	32.79	N
ATOM	1060	CA	ARG	629	8.051	95.067	4.898	1.00	34.31	C
ATOM	1061	C	ARG	629	8.588	94.118	3.822	1.00	30.81	C
ATOM	1062	O	ARG	629	8.919	94.556	2.723	1.00	36.74	O
ATOM	1063	CB	ARG	629	9.209	95.784	5.592	1.00	38.79	C
ATOM	1064	CG	ARG	629	10.042	94.861	6.431	1.00	47.03	C
ATOM	1065	CD	ARG	629	11.399	95.422	6.687	1.00	49.94	C
ATOM	1066	NE	ARG	629	12.324	94.336	6.978	1.00	58.97	N
ATOM	1067	CZ	ARG	629	13.603	94.339	6.633	1.00	65.16	C
ATOM	1068	NH1	ARG	629	14.109	95.379	5.985	1.00	70.46	N
ATOM	1069	NH2	ARG	629	14.378	93.314	6.952	1.00	68.49	N
ATOM	1070	N	MET	630	8.655	92.828	4.110	1.00	25.21	N
ATOM	1071	CA	MET	630	9.171	91.879	3.134	1.00	23.72	C
ATOM	1072	C	MET	630	8.061	91.297	2.268	1.00	24.23	C
ATOM	1073	O	MET	630	8.346	90.631	1.270	1.00	25.88	O
ATOM	1074	CB	MET	630	9.905	90.735	3.828	1.00	25.55	C
ATOM	1075	CG	MET	630	11.078	91.153	4.687	1.00	28.67	C
ATOM	1076	SD	MET	630	11.844	89.733	5.470	1.00	30.08	S
ATOM	1077	CE	MET	630	13.015	89.228	4.233	1.00	33.64	C
ATOM	1078	N	SER	631	6.811	91.617	2.606	1.00	23.98	N
ATOM	1079	CA	SER	631	5.634	91.098	1.906	1.00	24.27	C
ATOM	1080	C	SER	631	5.076	91.919	0.751	1.00	26.26	C
ATOM	1081	O	SER	631	4.010	91.584	0.210	1.00	21.34	O
ATOM	1082	CB	SER	631	4.504	90.879	2.911	1.00	22.54	C
ATOM	1083	OG	SER	631	4.923	90.050	3.983	1.00	24.17	O
ATOM	1084	N	ARG	632	5.797	92.967	0.357	1.00	29.47	N
ATOM	1085	CA	ARG	632	5.348	93.865	−0.711	1.00	27.92	C
ATOM	1086	C	ARG	632	4.990	93.255	−2.075	1.00	24.00	C
ATOM	1087	O	ARG	632	4.013	93.653	−2.698	1.00	19.20	O
ATOM	1088	CB	ARG	632	6.350	95.003	−0.869	1.00	32.62	C
ATOM	1089	CG	ARG	632	6.093	96.167	0.064	1.00	38.66	C
ATOM	1090	CD	ARG	632	7.198	97.197	−0.041	1.00	48.62	C
ATOM	1091	NE	ARG	632	8.267	96.958	0.932	1.00	58.66	N
ATOM	1092	CZ	ARG	632	9.535	97.343	0.786	1.00	61.73	C
ATOM	1093	NH1	ARG	632	9.927	97.984	−0.317	1.00	62.36	N
ATOM	1094	NH2	ARG	632	10.396	97.139	1.779	1.00	60.51	N
ATOM	1095	N	ASN	633	5.754	92.267	−2.520	1.00	22.34	N
ATOM	1096	CA	ASN	633	5.501	91.630	−3.812	1.00	23.39	C
ATOM	1097	C	ASN	633	4.823	90.274	−3.673	1.00	22.81	C
ATOM	1098	O	ASN	633	4.613	89.592	−4.665	1.00	20.30	O
ATOM	1099	CB	ASN	633	6.815	91.430	−4.583	1.00	19.08	C
ATOM	1100	CG	ASN	633	7.352	92.708	−5.169	1.00	17.97	C
ATOM	1101	OD1	ASN	633	6.663	93.724	−5.197	1.00	12.49	O
ATOM	1102	ND2	ASN	633	8.602	92.667	−5.644	1.00	17.03	N

TABLE 8-continued

Atomic Coordinates for Residues of a Crystal of murine VWF-A1 (SEQ ID NO: 10).

ATOM	1103	N	PHE	634	4.494	89.889	−2.445	1.00	22.28	N
ATOM	1104	CA	PHE	634	3.878	88.603	−2.182	1.00	19.74	C
ATOM	1105	C	PHE	634	2.821	88.172	−3.190	1.00	18.80	C
ATOM	1106	O	PHE	634	2.994	87.142	−3.849	1.00	19.49	O
ATOM	1107	CB	PHE	634	3.303	88.536	−0.756	1.00	14.84	C
ATOM	1108	CG	PHE	634	2.736	87.184	−0.414	1.00	11.94	C
ATOM	1109	CD1	PHE	634	3.583	86.149	−0.048	1.00	4.10	C
ATOM	1110	CD2	PHE	634	1.373	86.920	−0.576	1.00	11.23	C
ATOM	1111	CE1	PHE	634	3.095	84.880	0.136	1.00	5.83	C
ATOM	1112	CE2	PHE	634	0.858	85.645	−0.393	1.00	10.88	C
ATOM	1113	CZ	PHE	634	1.719	84.617	−0.040	1.00	9.89	C
ATOM	1114	N	VAL	635	1.751	88.942	−3.365	1.00	18.69	N
ATOM	1115	CA	VAL	635	0.707	88.514	−4.314	1.00	21.28	C
ATOM	1116	C	VAL	635	1.214	88.374	−5.751	1.00	21.28	C
ATOM	1117	O	VAL	635	0.803	87.478	−6.480	1.00	22.82	O
ATOM	1118	CB	VAL	635	−0.500	89.440	−4.280	1.00	20.51	C
ATOM	1119	CG1	VAL	635	−1.510	89.022	−5.316	1.00	25.23	C
ATOM	1120	CG2	VAL	635	−1.129	89.383	−2.922	1.00	23.14	C
ATOM	1121	N	ARG	636	2.160	89.227	−6.111	1.00	18.25	N
ATOM	1122	CA	ARG	636	2.778	89.239	−7.420	1.00	13.92	C
ATOM	1123	C	ARG	636	3.522	87.936	−7.677	1.00	15.53	C
ATOM	1124	O	ARG	636	3.419	87.366	−8.760	1.00	19.16	O
ATOM	1125	CB	ARG	636	3.719	90.446	−7.480	1.00	13.52	C
ATOM	1126	CG	ARG	636	2.923	91.760	−7.492	1.00	21.06	C
ATOM	1127	CD	ARG	636	3.742	92.986	−7.115	1.00	31.89	C
ATOM	1128	NE	ARG	636	3.059	94.231	−7.497	1.00	34.04	N
ATOM	1129	CZ	ARG	636	2.973	95.330	−6.747	1.00	33.28	C
ATOM	1130	NH1	ARG	636	3.523	95.373	−5.545	1.00	32.30	N
ATOM	1131	NH2	ARG	636	2.396	96.421	−7.233	1.00	35.94	N
ATOM	1132	N	TYR	637	4.249	87.439	−6.683	1.00	12.24	N
ATOM	1133	CA	TYR	637	4.969	86.187	−6.837	1.00	13.29	C
ATOM	1134	C	TYR	637	3.984	85.046	−6.970	1.00	15.41	C
ATOM	1135	O	TYR	637	4.176	84.141	−7.789	1.00	17.67	O
ATOM	1136	CB	TYR	637	5.912	85.942	−5.654	1.00	16.02	C
ATOM	1137	CG	TYR	637	7.003	86.987	−5.572	1.00	17.66	C
ATOM	1138	CD1	TYR	637	7.556	87.512	−6.726	1.00	23.47	C
ATOM	1139	CD2	TYR	637	7.438	87.486	−4.356	1.00	23.35	C
ATOM	1140	CE1	TYR	637	8.507	88.512	−6.684	1.00	24.21	C
ATOM	1141	CE2	TYR	637	8.398	88.493	−4.296	1.00	25.27	C
ATOM	1142	CZ	TYR	637	8.929	89.000	−5.471	1.00	27.11	C
ATOM	1143	OH	TYR	637	9.897	89.989	−5.446	1.00	27.67	O
ATOM	1144	N	VAL	638	2.892	85.114	−6.212	1.00	17.90	N
ATOM	1145	CA	VAL	638	1.866	84.073	−6.264	1.00	15.71	C
ATOM	1146	C	VAL	638	1.110	84.107	−7.591	1.00	16.42	C
ATOM	1147	O	VAL	638	0.730	83.057	−8.121	1.00	13.74	O
ATOM	1148	CB	VAL	638	0.879	84.189	−5.070	1.00	14.51	C
ATOM	1149	CG1	VAL	638	−0.224	83.196	−5.201	1.00	10.64	C
ATOM	1150	CG2	VAL	638	1.623	83.907	−3.762	1.00	15.68	C
ATOM	1151	N	GLN	639	0.858	85.306	−8.122	1.00	17.70	N
ATOM	1152	CA	GLN	639	0.173	85.418	−9.401	1.00	19.16	C
ATOM	1153	C	GLN	639	1.132	84.973	−10.494	1.00	20.06	C
ATOM	1154	O	GLN	639	0.705	84.381	−11.500	1.00	21.31	O
ATOM	1155	CB	GLN	639	−0.280	86.842	−9.661	1.00	24.29	C
ATOM	1156	CG	GLN	639	−1.377	87.339	−8.758	1.00	33.20	C
ATOM	1157	CD	GLN	639	−1.509	88.855	−8.824	1.00	42.75	C
ATOM	1158	OE1	GLN	639	−0.534	89.573	−9.095	1.00	47.34	O
ATOM	1159	NE2	GLN	639	−2.709	89.353	−8.572	1.00	47.82	N
ATOM	1160	N	GLY	640	2.428	85.212	−10.272	1.00	19.08	N
ATOM	1161	CA	GLY	640	3.465	84.789	−11.212	1.00	16.51	C
ATOM	1162	C	GLY	640	3.515	83.268	−11.342	1.00	16.94	C
ATOM	1163	O	GLY	640	3.492	82.740	−12.454	1.00	19.48	O
ATOM	1164	N	LEU	641	3.553	82.551	−10.221	1.00	16.74	N
ATOM	1165	CA	LEU	641	3.579	81.088	−10.252	1.00	13.10	C
ATOM	1166	C	LEU	641	2.269	80.555	−10.792	1.00	13.33	C
ATOM	1167	O	LEU	641	2.206	79.465	−11.355	1.00	18.15	O
ATOM	1168	CB	LEU	641	3.802	80.530	−8.852	1.00	14.38	C
ATOM	1169	CG	LEU	641	5.198	80.681	−8.238	1.00	16.65	C
ATOM	1170	CD1	LEU	641	5.139	80.462	−6.733	1.00	18.86	C
ATOM	1171	CD2	LEU	641	6.206	79.737	−8.886	1.00	6.08	C
ATOM	1172	N	LYS	642	1.205	81.311	−10.605	1.00	14.11	N
ATOM	1173	CA	LYS	642	−0.101	80.898	−11.096	1.00	16.06	C
ATOM	1174	C	LYS	642	−0.150	80.870	−12.623	1.00	16.97	C
ATOM	1175	O	LYS	642	−0.809	80.019	−13.210	1.00	19.62	O

TABLE 8-continued

Atomic Coordinates for Residues of a Crystal of murine VWF-A1 (SEQ ID NO: 10).

ATOM	1176	CB	LYS	642	−1.169	81.847	−10.555	1.00	16.72	C
ATOM	1177	CG	LYS	642	−2.558	81.585	−11.078	1.00	18.86	C
ATOM	1178	CD	LYS	642	−3.085	82.761	−11.881	1.00	14.53	C
ATOM	1179	CE	LYS	642	−4.522	82.498	−12.267	1.00	25.13	C
ATOM	1180	NZ	LYS	642	−5.053	83.581	−13.128	1.00	33.85	N
ATOM	1181	N	LYS	643	0.517	81.829	−13.256	1.00	17.23	N
ATOM	1182	CA	LYS	643	0.554	81.933	−14.714	1.00	15.33	C
ATOM	1183	C	LYS	643	1.409	80.845	−15.322	1.00	14.03	C
ATOM	1184	O	LYS	643	1.183	80.439	−16.464	1.00	13.60	O
ATOM	1185	CB	LYS	643	1.127	83.281	−15.127	1.00	16.90	C
ATOM	1186	CG	LYS	643	0.301	84.453	−14.692	1.00	23.60	C
ATOM	1187	CD	LYS	643	0.990	85.751	−15.080	1.00	29.59	C
ATOM	1188	CE	LYS	643	0.321	86.922	−14.390	1.00	34.30	C
ATOM	1189	NZ	LYS	643	1.216	88.106	−14.326	1.00	35.59	N
ATOM	1190	N	LYS	644	2.442	80.431	−14.598	1.00	15.71	N
ATOM	1191	CA	LYS	644	3.321	79.373	−15.059	1.00	15.56	C
ATOM	1192	C	LYS	644	2.720	78.041	−14.644	1.00	13.62	C
ATOM	1193	O	LYS	644	3.351	77.009	−14.825	1.00	16.26	O
ATOM	1194	CB	LYS	644	4.737	79.517	−14.466	1.00	19.22	C
ATOM	1195	CG	LYS	644	5.710	80.398	−15.264	1.00	22.27	C
ATOM	1196	CD	LYS	644	7.123	80.272	−14.690	1.00	24.71	C
ATOM	1197	CE	LYS	644	8.123	81.299	−15.260	1.00	31.85	C
ATOM	1198	NZ	LYS	644	9.118	81.871	−14.243	1.00	23.70	N
ATOM	1199	N	LYS	645	1.502	78.076	−14.098	1.00	10.95	N
ATOM	1200	CA	LYS	645	0.761	76.876	−13.644	1.00	14.53	C
ATOM	1201	C	LYS	645	1.425	76.068	−12.527	1.00	15.63	C
ATOM	1202	O	LYS	645	1.324	74.833	−12.481	1.00	10.70	O
ATOM	1203	CB	LYS	645	0.397	75.957	−14.803	1.00	14.90	C
ATOM	1204	CG	LYS	645	−1.087	75.949	−15.174	1.00	16.56	C
ATOM	1205	CD	LYS	645	−1.866	75.078	−14.228	1.00	19.52	C
ATOM	1206	CE	LYS	645	−3.242	75.666	−13.949	1.00	28.98	C
ATOM	1207	NZ	LYS	645	−4.386	74.911	−14.522	1.00	29.12	N
ATOM	1208	N	VAL	646	2.095	76.784	−11.630	1.00	13.98	N
ATOM	1209	CA	VAL	646	2.745	76.182	−10.481	1.00	17.17	C
ATOM	1210	C	VAL	646	1.723	76.303	−9.379	1.00	17.08	C
ATOM	1211	O	VAL	646	1.333	77.413	−9.028	1.00	15.83	O
ATOM	1212	CB	VAL	646	4.004	76.952	−10.065	1.00	15.22	C
ATOM	1213	CG1	VAL	646	4.628	76.308	−8.805	1.00	14.08	C
ATOM	1214	CG2	VAL	646	4.980	76.955	−11.204	1.00	10.59	C
ATOM	1215	N	ILE	647	1.247	75.160	−8.895	1.00	17.86	N
ATOM	1216	CA	ILE	647	0.235	75.107	−7.852	1.00	15.74	C
ATOM	1217	C	ILE	647	0.858	75.303	−6.464	1.00	15.68	C
ATOM	1218	O	ILE	647	1.785	74.592	−6.086	1.00	17.61	O
ATOM	1219	CB	ILE	647	−0.514	73.765	−7.947	1.00	14.07	C
ATOM	1220	CG1	ILE	647	−1.094	73.600	−9.364	1.00	17.76	C
ATOM	1221	CG2	ILE	647	−1.620	73.665	−6.896	1.00	12.37	C
ATOM	1222	CD1	ILE	647	−1.913	74.795	−9.865	1.00	11.46	C
ATOM	1223	N	VAL	648	0.400	76.298	−5.722	1.00	16.62	N
ATOM	1224	CA	VAL	648	0.969	76.519	−4.387	1.00	16.18	C
ATOM	1225	C	VAL	648	0.004	75.932	−3.365	1.00	15.96	C
ATOM	1226	O	VAL	648	−1.158	76.338	−3.284	1.00	14.76	O
ATOM	1227	CB	VAL	648	1.258	78.035	−4.079	1.00	12.13	C
ATOM	1228	CG1	VAL	648	2.023	78.179	−2.775	1.00	9.31	C
ATOM	1229	CG2	VAL	648	2.078	78.678	−5.223	1.00	11.58	C
ATOM	1230	N	ILE	649	0.456	74.906	−2.659	1.00	15.66	N
ATOM	1231	CA	ILE	649	−0.369	74.276	−1.644	1.00	16.78	C
ATOM	1232	C	ILE	649	0.222	74.671	−0.306	1.00	17.79	C
ATOM	1233	O	ILE	649	1.223	74.095	0.124	1.00	18.08	O
ATOM	1234	CB	ILE	649	−0.376	72.747	−1.769	1.00	14.88	C
ATOM	1235	CG1	ILE	649	−0.873	72.352	−3.159	1.00	16.15	C
ATOM	1236	CG2	ILE	649	−1.297	72.142	−0.708	1.00	16.62	C
ATOM	1237	CD1	ILE	649	−0.925	70.873	−3.418	1.00	17.42	C
ATOM	1238	N	PRO	650	−0.351	75.709	0.334	1.00	16.08	N
ATOM	1239	CA	PRO	650	0.076	76.238	1.627	1.00	12.67	C
ATOM	1240	C	PRO	650	−0.300	75.297	2.775	1.00	15.98	C
ATOM	1241	O	PRO	650	−1.478	74.921	2.940	1.00	13.96	O
ATOM	1242	CB	PRO	650	−0.737	77.512	1.764	1.00	9.54	C
ATOM	1243	CG	PRO	650	−1.475	77.687	0.465	1.00	13.54	C
ATOM	1244	CD	PRO	650	−1.626	76.326	−0.068	1.00	16.46	C
ATOM	1245	N	VAL	651	0.693	74.893	3.553	1.00	14.32	N
ATOM	1246	CA	VAL	651	0.422	74.040	4.697	1.00	14.36	C
ATOM	1247	C	VAL	651	0.728	74.922	5.911	1.00	12.69	C
ATOM	1248	O	VAL	651	1.871	75.307	6.129	1.00	15.37	O

TABLE 8-continued

Atomic Coordinates for Residues of a Crystal of murine VWF-A1 (SEQ ID NO: 10).

ATOM	1249	CB	VAL	651	1.293	72.782	4.691	1.00	14.81	C
ATOM	1250	CG1	VAL	651	0.942	71.918	5.884	1.00	15.50	C
ATOM	1251	CG2	VAL	651	1.075	72.004	3.392	1.00	10.07	C
ATOM	1252	N	GLY	652	−0.307	75.355	6.617	1.00	9.84	N
ATOM	1253	CA	GLY	652	−0.102	76.201	7.776	1.00	12.11	C
ATOM	1254	C	GLY	652	0.074	75.324	8.991	1.00	11.33	C
ATOM	1255	O	GLY	652	−0.778	74.503	9.244	1.00	17.62	O
ATOM	1256	N	ILE	653	1.150	75.492	9.742	1.00	13.97	N
ATOM	1257	CA	ILE	653	1.397	74.679	10.931	1.00	18.24	C
ATOM	1258	C	ILE	653	1.481	75.547	12.197	1.00	21.88	C
ATOM	1259	O	ILE	653	2.468	76.273	12.417	1.00	19.90	O
ATOM	1260	CB	ILE	653	2.728	73.937	10.804	1.00	22.12	C
ATOM	1261	CG1	ILE	653	2.805	73.167	9.478	1.00	18.02	C
ATOM	1262	CG2	ILE	653	2.957	73.057	12.034	1.00	18.87	C
ATOM	1263	CD1	ILE	653	4.217	72.868	9.064	1.00	14.43	C
ATOM	1264	N	GLY	654	0.445	75.491	13.023	1.00	17.34	N
ATOM	1265	CA	GLY	654	0.480	76.279	14.245	1.00	18.39	C
ATOM	1266	C	GLY	654	−0.111	77.682	14.187	1.00	20.92	C
ATOM	1267	O	GLY	654	−0.373	78.214	13.102	1.00	24.39	O
ATOM	1268	N	PRO	655	−0.264	78.337	15.363	1.00	20.00	N
ATOM	1269	CA	PRO	655	−0.806	79.684	15.601	1.00	15.28	C
ATOM	1270	C	PRO	655	−0.034	80.794	14.911	1.00	16.41	C
ATOM	1271	O	PRO	655	−0.588	81.825	14.552	1.00	18.28	O
ATOM	1272	CB	PRO	655	−0.642	79.852	17.116	1.00	12.24	C
ATOM	1273	CG	PRO	655	−0.672	78.460	17.630	1.00	13.05	C
ATOM	1274	CD	PRO	655	0.153	77.719	16.635	1.00	14.07	C
ATOM	1275	N	HIS	656	1.272	80.622	14.794	1.00	16.41	N
ATOM	1276	CA	HIS	656	2.082	81.667	14.194	1.00	18.52	C
ATOM	1277	C	HIS	656	2.109	81.703	12.664	1.00	17.35	C
ATOM	1278	O	HIS	656	2.603	82.672	12.084	1.00	17.65	O
ATOM	1279	CB	HIS	656	3.492	81.676	14.815	1.00	16.60	C
ATOM	1280	CG	HIS	656	3.498	82.026	16.283	1.00	18.39	C
ATOM	1281	ND1	HIS	656	3.392	81.077	17.277	1.00	18.56	N
ATOM	1282	CD2	HIS	656	3.554	83.224	16.915	1.00	16.46	C
ATOM	1283	CE1	HIS	656	3.376	81.672	18.451	1.00	13.19	C
ATOM	1284	NE2	HIS	656	3.474	82.972	18.258	1.00	16.12	N
ATOM	1285	N	ALA	657	1.504	80.703	12.027	1.00	17.30	N
ATOM	1286	CA	ALA	657	1.459	80.616	10.564	1.00	18.06	C
ATOM	1287	C	ALA	657	0.729	81.810	9.961	1.00	16.82	C
ATOM	1288	O	ALA	657	−0.300	82.248	10.464	1.00	19.76	O
ATOM	1289	CB	ALA	657	0.808	79.297	10.121	1.00	13.90	C
ATOM	1290	N	ASN	658	1.277	82.340	8.877	1.00	19.53	N
ATOM	1291	CA	ASN	658	0.699	83.502	8.226	1.00	16.37	C
ATOM	1292	C	ASN	658	−0.569	83.144	7.494	1.00	18.85	C
ATOM	1293	O	ASN	658	−0.575	82.964	6.284	1.00	20.50	O
ATOM	1294	CB	ASN	658	1.704	84.142	7.276	1.00	11.24	C
ATOM	1295	CG	ASN	658	1.282	85.534	6.844	1.00	11.66	C
ATOM	1296	OD1	ASN	658	0.098	85.829	6.766	1.00	14.75	O
ATOM	1297	ND2	ASN	658	2.251	86.396	6.563	1.00	16.32	N
ATOM	1298	N	LEU	659	−1.648	83.035	8.251	1.00	20.82	N
ATOM	1299	CA	LEU	659	−2.943	82.704	7.700	1.00	19.30	C
ATOM	1300	C	LEU	659	−3.460	83.699	6.664	1.00	20.85	C
ATOM	1301	O	LEU	659	−4.230	83.317	5.782	1.00	22.69	O
ATOM	1302	CB	LEU	659	−3.950	82.569	8.824	1.00	17.74	C
ATOM	1303	CG	LEU	659	−4.579	81.198	8.897	1.00	21.02	C
ATOM	1304	CD1	LEU	659	−3.505	80.188	9.248	1.00	24.59	C
ATOM	1305	CD2	LEU	659	−5.665	81.212	9.935	1.00	27.06	C
ATOM	1306	N	LYS	660	−3.078	84.968	6.782	1.00	22.50	N
ATOM	1307	CA	LYS	660	−3.520	85.982	5.822	1.00	23.11	C
ATOM	1308	C	LYS	660	−2.945	85.639	4.452	1.00	24.26	C
ATOM	1309	O	LYS	660	−3.663	85.697	3.448	1.00	27.91	O
ATOM	1310	CB	LYS	660	−3.068	87.361	6.251	1.00	21.43	C
ATOM	1311	N	GLN	661	−1.667	85.240	4.420	1.00	21.76	N
ATOM	1312	CA	GLN	661	−0.991	84.844	3.183	1.00	17.85	C
ATOM	1313	C	GLN	661	−1.570	83.531	2.688	1.00	18.55	C
ATOM	1314	O	GLN	661	−1.828	83.372	1.499	1.00	23.06	O
ATOM	1315	CB	GLN	661	0.525	84.730	3.377	1.00	12.86	C
ATOM	1316	CG	GLN	661	1.237	86.102	3.366	1.00	10.63	C
ATOM	1317	CD	GLN	661	2.755	86.013	3.544	1.00	14.73	C
ATOM	1318	OE1	GLN	661	3.287	84.965	3.905	1.00	17.21	O
ATOM	1319	NE2	GLN	661	3.451	87.118	3.305	1.00	15.89	N
ATOM	1320	N	ILE	662	−1.850	82.613	3.607	1.00	17.78	N
ATOM	1321	CA	ILE	662	−2.438	81.336	3.237	1.00	15.00	C

TABLE 8-continued

Atomic Coordinates for Residues of a Crystal of murine VWF-A1 (SEQ ID NO: 10).

ATOM	1322	C	ILE	662	−3.775	81.610	2.537	1.00	19.04	C
ATOM	1323	O	ILE	662	−4.050	81.031	1.487	1.00	21.90	O
ATOM	1324	CB	ILE	662	−2.580	80.411	4.489	1.00	11.85	C
ATOM	1325	CG1	ILE	662	−1.196	80.040	4.995	1.00	11.79	C
ATOM	1326	CG2	ILE	662	−3.337	79.126	4.196	1.00	7.88	C
ATOM	1327	CD1	ILE	662	−1.191	79.084	6.171	1.00	13.32	C
ATOM	1328	N	ARG	663	−4.558	82.560	3.047	1.00	20.47	N
ATOM	1329	CA	ARG	663	−5.850	82.874	2.436	1.00	20.44	C
ATOM	1330	C	ARG	663	−5.704	83.569	1.078	1.00	19.11	C
ATOM	1331	O	ARG	663	−6.548	83.416	0.195	1.00	19.37	O
ATOM	1332	CB	ARG	663	−6.733	83.701	3.390	1.00	23.96	C
ATOM	1333	CG	ARG	663	−7.123	82.987	4.686	1.00	32.86	C
ATOM	1334	CD	ARG	663	−7.800	81.612	4.456	1.00	41.17	C
ATOM	1335	NE	ARG	663	−7.818	80.785	5.672	1.00	43.59	N
ATOM	1336	CZ	ARG	663	−8.042	79.471	5.708	1.00	44.43	C
ATOM	1337	NH1	ARG	663	−8.287	78.791	4.604	1.00	40.35	N
ATOM	1338	NH2	ARG	663	−7.942	78.818	6.853	1.00	47.33	N
ATOM	1339	N	LEU	664	−4.641	84.338	0.911	1.00	20.67	N
ATOM	1340	CA	LEU	664	−4.400	85.016	−0.351	1.00	23.77	C
ATOM	1341	C	LEU	664	−4.006	83.990	−1.425	1.00	24.53	C
ATOM	1342	O	LEU	664	−4.470	84.066	−2.570	1.00	25.26	O
ATOM	1343	CB	LEU	664	−3.287	86.048	−0.200	1.00	22.42	C
ATOM	1344	CG	LEU	664	−3.695	87.458	0.188	1.00	23.55	C
ATOM	1345	CD1	LEU	664	−2.434	88.240	0.463	1.00	22.20	C
ATOM	1346	CD2	LEU	664	−4.517	88.113	−0.910	1.00	21.42	C
ATOM	1347	N	ILE	665	−3.147	83.040	−1.053	1.00	23.53	N
ATOM	1348	CA	ILE	665	−2.697	82.009	−1.970	1.00	21.00	C
ATOM	1349	C	ILE	665	−3.895	81.209	−2.462	1.00	22.09	C
ATOM	1350	O	ILE	665	−4.064	81.029	−3.659	1.00	24.56	O
ATOM	1351	CB	ILE	665	−1.680	81.078	−1.316	1.00	17.66	C
ATOM	1352	CG1	ILE	665	−0.391	81.841	−1.015	1.00	15.92	C
ATOM	1353	CG2	ILE	665	−1.375	79.913	−2.234	1.00	18.28	C
ATOM	1354	CD1	ILE	665	0.629	81.031	−0.194	1.00	6.96	C
ATOM	1355	N	GLU	666	−4.732	80.747	−1.549	1.00	23.35	N
ATOM	1356	CA	GLU	666	−5.917	79.983	−1.918	1.00	19.08	C
ATOM	1357	C	GLU	666	−6.813	80.616	−2.967	1.00	19.61	C
ATOM	1358	O	GLU	666	−7.341	79.920	−3.827	1.00	18.02	O
ATOM	1359	CB	GLU	666	−6.784	79.754	−0.704	1.00	17.71	C
ATOM	1360	CG	GLU	666	−6.367	78.618	0.172	1.00	26.16	C
ATOM	1361	CD	GLU	666	−7.515	78.164	1.049	1.00	28.97	C
ATOM	1362	OE1	GLU	666	−7.910	78.950	1.944	1.00	22.27	O
ATOM	1363	OE2	GLU	666	−8.030	77.036	0.815	1.00	30.15	O
ATOM	1364	N	LYS	667	−7.009	81.928	−2.878	1.00	21.77	N
ATOM	1365	CA	LYS	667	−7.908	82.604	−3.800	1.00	27.64	C
ATOM	1366	C	LYS	667	−7.336	83.042	−5.140	1.00	28.79	C
ATOM	1367	O	LYS	667	−8.067	83.548	−5.999	1.00	33.07	O
ATOM	1368	CB	LYS	667	−8.613	83.773	−3.108	1.00	29.02	C
ATOM	1369	CG	LYS	667	−7.764	84.976	−2.848	1.00	34.25	C
ATOM	1370	CD	LYS	667	−8.600	86.097	−2.236	1.00	45.36	C
ATOM	1371	CE	LYS	667	−7.746	87.344	−1.955	1.00	55.46	C
ATOM	1372	NZ	LYS	667	−8.130	88.070	−0.688	1.00	58.49	N
ATOM	1373	N	GLN	668	−6.042	82.833	−5.329	1.00	29.18	N
ATOM	1374	CA	GLN	668	−5.370	83.189	−6.570	1.00	27.43	C
ATOM	1375	C	GLN	668	−5.720	82.143	−7.648	1.00	27.44	C
ATOM	1376	O	GLN	668	−5.795	82.459	−8.834	1.00	29.48	O
ATOM	1377	CB	GLN	668	−3.856	83.236	−6.324	1.00	24.44	C
ATOM	1378	CG	GLN	668	−3.199	84.560	−6.643	1.00	31.15	C
ATOM	1379	CD	GLN	668	−3.934	85.764	−6.071	1.00	33.50	C
ATOM	1380	OE1	GLN	668	−4.353	86.659	−6.818	1.00	32.99	O
ATOM	1381	NE2	GLN	668	−4.054	85.819	−4.747	1.00	30.05	N
ATOM	1382	N	ALA	669	−5.930	80.895	−7.231	1.00	23.35	N
ATOM	1383	CA	ALA	669	−6.275	79.816	−8.153	1.00	21.93	C
ATOM	1384	C	ALA	669	−6.968	78.683	−7.379	1.00	20.53	C
ATOM	1385	O	ALA	669	−6.590	78.375	−6.267	1.00	22.44	O
ATOM	1386	CB	ALA	669	−5.010	79.303	−8.854	1.00	21.51	C
ATOM	1387	N	PRO	670	−7.983	78.041	−7.977	1.00	22.07	N
ATOM	1388	CA	PRO	670	−8.697	76.959	−7.300	1.00	19.32	C
ATOM	1389	C	PRO	670	−7.916	75.769	−6.744	1.00	24.31	C
ATOM	1390	O	PRO	670	−8.338	75.182	−5.735	1.00	22.85	O
ATOM	1391	CB	PRO	670	−9.740	76.517	−8.344	1.00	19.25	C
ATOM	1392	CG	PRO	670	−9.251	77.058	−9.633	1.00	18.97	C
ATOM	1393	CD	PRO	670	−8.649	78.372	−9.251	1.00	22.64	C
ATOM	1394	N	GLU	671	−6.765	75.444	−7.332	1.00	21.90	N

TABLE 8-continued

Atomic Coordinates for Residues of a Crystal of murine VWF-A1 (SEQ ID NO: 10).

ATOM	1395	CA	GLU	671	−6.037	74.282	−6.872	1.00	21.53	C
ATOM	1396	C	GLU	671	−5.121	74.573	−5.724	1.00	18.85	C
ATOM	1397	O	GLU	671	−4.526	73.648	−5.152	1.00	17.02	O
ATOM	1398	CB	GLU	671	−5.237	73.622	−7.996	1.00	28.16	C
ATOM	1399	CG	GLU	671	−6.053	73.156	−9.167	1.00	30.85	C
ATOM	1400	CD	GLU	671	−6.052	74.163	−10.295	1.00	40.33	C
ATOM	1401	OE1	GLU	671	−5.922	75.389	−10.033	1.00	37.89	O
ATOM	1402	OE2	GLU	671	−6.181	73.718	−11.456	1.00	49.39	O
ATOM	1403	N	ASN	672	−4.915	75.849	−5.440	1.00	16.70	N
ATOM	1404	CA	ASN	672	−4.035	76.229	−4.324	1.00	20.34	C
ATOM	1405	C	ASN	672	−4.755	75.885	−3.007	1.00	18.55	C
ATOM	1406	O	ASN	672	−5.136	76.780	−2.248	1.00	18.65	O
ATOM	1407	CB	ASN	672	−3.751	77.739	−4.359	1.00	19.53	C
ATOM	1408	CG	ASN	672	−2.994	78.186	−5.601	1.00	15.33	C
ATOM	1409	OD1	ASN	672	−2.466	77.384	−6.369	1.00	12.87	O
ATOM	1410	ND2	ASN	672	−2.914	79.494	−5.778	1.00	17.27	N
ATOM	1411	N	LYS	673	−4.986	74.601	−2.761	1.00	17.76	N
ATOM	1412	CA	LYS	673	−5.708	74.178	−1.568	1.00	21.07	C
ATOM	1413	C	LYS	673	−4.820	74.299	−0.335	1.00	20.99	C
ATOM	1414	O	LYS	673	−3.655	73.896	−0.360	1.00	26.08	O
ATOM	1415	CB	LYS	673	−6.222	72.732	−1.710	1.00	19.00	C
ATOM	1416	CG	LYS	673	−7.480	72.469	−0.858	1.00	30.07	C
ATOM	1417	CD	LYS	673	−7.457	71.176	−0.047	1.00	30.38	C
ATOM	1418	CE	LYS	673	−7.751	69.983	−0.913	1.00	36.80	C
ATOM	1419	NZ	LYS	673	−7.645	68.682	−0.181	1.00	45.16	N
ATOM	1420	N	ALA	674	−5.357	74.891	0.727	1.00	18.75	N
ATOM	1421	CA	ALA	674	−4.613	75.043	1.959	1.00	15.72	C
ATOM	1422	C	ALA	674	−4.928	73.914	2.916	1.00	18.41	C
ATOM	1423	O	ALA	674	−6.047	73.370	2.935	1.00	12.37	O
ATOM	1424	CB	ALA	674	−4.938	76.362	2.607	1.00	14.82	C
ATOM	1425	N	PHE	675	−3.916	73.577	3.716	1.00	20.72	N
ATOM	1426	CA	PHE	675	−4.021	72.560	4.751	1.00	17.35	C
ATOM	1427	C	PHE	675	−3.565	73.235	6.032	1.00	13.94	C
ATOM	1428	O	PHE	675	−2.414	73.594	6.162	1.00	16.79	O
ATOM	1429	CB	PHE	675	−3.148	71.357	4.421	1.00	17.30	C
ATOM	1430	CG	PHE	675	−3.667	70.547	3.276	1.00	16.73	C
ATOM	1431	CD1	PHE	675	−3.357	70.890	1.961	1.00	18.39	C
ATOM	1432	CD2	PHE	675	−4.502	69.469	3.501	1.00	11.98	C
ATOM	1433	CE1	PHE	675	−3.880	70.177	0.880	1.00	11.40	C
ATOM	1434	CE2	PHE	675	−5.031	68.746	2.416	1.00	15.53	C
ATOM	1435	CZ	PHE	675	−4.717	69.111	1.108	1.00	13.44	C
ATOM	1436	N	VAL	676	−4.509	73.561	6.899	1.00	17.73	N
ATOM	1437	CA	VAL	676	−4.176	74.211	8.163	1.00	19.36	C
ATOM	1438	C	VAL	676	−4.128	73.145	9.260	1.00	20.91	C
ATOM	1439	O	VAL	676	−5.035	72.324	9.395	1.00	19.66	O
ATOM	1440	CB	VAL	676	−5.175	75.326	8.489	1.00	17.55	C
ATOM	1441	CG1	VAL	676	−4.779	76.031	9.769	1.00	18.65	C
ATOM	1442	CG2	VAL	676	−5.219	76.328	7.325	1.00	19.14	C
ATOM	1443	N	LEU	677	−2.994	73.100	9.952	1.00	23.37	N
ATOM	1444	CA	LEU	677	−2.740	72.122	11.003	1.00	21.69	C
ATOM	1445	C	LEU	677	−2.362	72.830	12.284	1.00	17.44	C
ATOM	1446	O	LEU	677	−1.870	73.965	12.263	1.00	11.94	O
ATOM	1447	CB	LEU	677	−1.597	71.184	10.603	1.00	25.46	C
ATOM	1448	CG	LEU	677	−1.766	70.099	9.540	1.00	25.42	C
ATOM	1449	CD1	LEU	677	−2.269	70.624	8.236	1.00	23.58	C
ATOM	1450	CD2	LEU	677	−0.422	69.495	9.346	1.00	30.67	C
ATOM	1451	N	SER	678	−2.591	72.153	13.403	1.00	15.75	N
ATOM	1452	CA	SER	678	−2.279	72.728	14.704	1.00	18.74	C
ATOM	1453	C	SER	678	−0.805	72.690	15.096	1.00	15.98	C
ATOM	1454	O	SER	678	−0.305	73.594	15.775	1.00	16.52	O
ATOM	1455	CB	SER	678	−3.143	72.085	15.783	1.00	17.24	C
ATOM	1456	OG	SER	678	−4.495	72.476	15.621	1.00	23.02	O
ATOM	1457	N	SER	679	−0.095	71.679	14.621	1.00	15.13	N
ATOM	1458	CA	SER	679	1.305	71.550	14.954	1.00	16.64	C
ATOM	1459	C	SER	679	1.891	70.526	13.993	1.00	16.28	C
ATOM	1460	O	SER	679	1.152	69.830	13.284	1.00	16.75	O
ATOM	1461	CB	SER	679	1.424	71.025	16.389	1.00	20.41	C
ATOM	1462	OG	SER	679	0.924	69.683	16.472	1.00	15.18	O
ATOM	1463	N	VAL	680	3.212	70.389	14.023	1.00	14.70	N
ATOM	1464	CA	VAL	680	3.881	69.426	13.165	1.00	18.08	C
ATOM	1465	C	VAL	680	3.441	67.999	13.506	1.00	21.23	C
ATOM	1466	O	VAL	680	3.598	67.092	12.692	1.00	22.59	O
ATOM	1467	CB	VAL	680	5.421	69.505	13.291	1.00	15.16	C

TABLE 8-continued

Atomic Coordinates for Residues of a Crystal of murine VWF-A1 (SEQ ID NO: 10).

ATOM	1468	CG1	VAL	680	5.953	70.852	12.792	1.00	14.14	C
ATOM	1469	CG2	VAL	680	5.844	69.260	14.728	1.00	15.78	C
ATOM	1470	N	ASP	681	2.845	67.803	14.680	1.00	23.61	N
ATOM	1471	CA	ASP	681	2.431	66.465	15.090	1.00	20.29	C
ATOM	1472	C	ASP	681	1.267	65.967	14.292	1.00	17.46	C
ATOM	1473	O	ASP	681	1.021	64.758	14.254	1.00	19.43	O
ATOM	1474	CB	ASP	681	2.118	66.404	16.586	1.00	22.71	C
ATOM	1475	CG	ASP	681	3.360	66.605	17.455	1.00	27.60	C
ATOM	1476	OD1	ASP	681	4.485	66.251	17.021	1.00	31.79	O
ATOM	1477	OD2	ASP	681	3.205	67.117	18.585	1.00	30.83	O
ATOM	1478	N	GLU	682	0.559	66.891	13.648	1.00	19.92	N
ATOM	1479	CA	GLU	682	−0.593	66.532	12.823	1.00	19.43	C
ATOM	1480	C	GLU	682	−0.246	66.200	11.373	1.00	16.94	C
ATOM	1481	O	GLU	682	−1.031	65.593	10.678	1.00	20.34	O
ATOM	1482	CB	GLU	682	−1.664	67.616	12.904	1.00	19.71	C
ATOM	1483	CG	GLU	682	−2.031	67.903	14.337	1.00	27.72	C
ATOM	1484	CD	GLU	682	−3.385	68.541	14.516	1.00	32.65	C
ATOM	1485	OE1	GLU	682	−3.806	69.338	13.643	1.00	32.50	O
ATOM	1486	OE2	GLU	682	−4.013	68.250	15.567	1.00	36.39	O
ATOM	1487	N	LEU	683	0.968	66.533	10.953	1.00	19.46	N
ATOM	1488	CA	LEU	683	1.430	66.270	9.591	1.00	18.53	C
ATOM	1489	C	LEU	683	1.230	64.820	9.159	1.00	19.54	C
ATOM	1490	O	LEU	683	0.759	64.567	8.050	1.00	21.33	O
ATOM	1491	CB	LEU	683	2.913	66.629	9.458	1.00	9.01	C
ATOM	1492	CG	LEU	683	3.212	68.119	9.485	1.00	11.07	C
ATOM	1493	CD1	LEU	683	4.725	68.380	9.521	1.00	6.20	C
ATOM	1494	CD2	LEU	683	2.581	68.729	8.268	1.00	9.94	C
ATOM	1495	N	GLU	684	1.577	63.879	10.027	1.00	17.82	N
ATOM	1496	CA	GLU	684	1.449	62.470	9.701	1.00	21.58	C
ATOM	1497	C	GLU	684	0.026	62.043	9.404	1.00	20.80	C
ATOM	1498	O	GLU	684	−0.204	61.216	8.521	1.00	21.28	O
ATOM	1499	CB	GLU	684	2.022	61.614	10.826	1.00	27.44	C
ATOM	1500	CG	GLU	684	1.799	60.112	10.601	1.00	42.61	C
ATOM	1501	CD	GLU	684	2.605	59.219	11.539	1.00	48.25	C
ATOM	1502	OE1	GLU	684	2.910	59.655	12.683	1.00	46.17	O
ATOM	1503	OE2	GLU	684	2.915	58.071	11.116	1.00	50.28	O
ATOM	1504	N	GLN	685	−0.914	62.632	10.133	1.00	22.35	N
ATOM	1505	CA	GLN	685	−2.349	62.353	10.005	1.00	25.85	C
ATOM	1506	C	GLN	685	−2.958	62.892	8.709	1.00	22.14	C
ATOM	1507	O	GLN	685	−4.089	62.579	8.381	1.00	25.03	O
ATOM	1508	CB	GLN	685	−3.117	63.008	11.162	1.00	25.71	C
ATOM	1509	CG	GLN	685	−2.414	62.982	12.509	1.00	34.50	C
ATOM	1510	CD	GLN	685	−3.192	63.753	13.571	1.00	41.31	C
ATOM	1511	OE1	GLN	685	−4.338	64.167	13.356	1.00	40.86	O
ATOM	1512	NE2	GLN	685	−2.565	63.957	14.726	1.00	45.33	N
ATOM	1513	N	GLN	686	−2.245	63.789	8.044	1.00	20.96	N
ATOM	1514	CA	GLN	686	−2.715	64.404	6.813	1.00	23.16	C
ATOM	1515	C	GLN	686	−1.882	64.075	5.583	1.00	19.32	C
ATOM	1516	O	GLN	686	−2.328	64.299	4.456	1.00	22.21	O
ATOM	1517	CB	GLN	686	−2.709	65.912	6.982	1.00	26.35	C
ATOM	1518	CG	GLN	686	−3.709	66.416	7.948	1.00	35.22	C
ATOM	1519	CD	GLN	686	−4.816	67.142	7.241	1.00	42.30	C
ATOM	1520	OE1	GLN	686	−4.941	68.365	7.350	1.00	45.54	O
ATOM	1521	NE2	GLN	686	−5.616	66.400	6.478	1.00	44.88	N
ATOM	1522	N	ARG	687	−0.683	63.557	5.803	1.00	14.84	N
ATOM	1523	CA	ARG	687	0.238	63.225	4.731	1.00	17.34	C
ATOM	1524	C	ARG	687	−0.364	62.511	3.524	1.00	17.95	C
ATOM	1525	O	ARG	687	−0.248	62.982	2.402	1.00	18.16	O
ATOM	1526	CB	ARG	687	1.405	62.429	5.303	1.00	12.76	C
ATOM	1527	CG	ARG	687	2.512	62.076	4.320	1.00	12.31	C
ATOM	1528	CD	ARG	687	2.812	60.589	4.398	1.00	15.47	C
ATOM	1529	NE	ARG	687	2.836	60.134	5.786	1.00	27.33	N
ATOM	1530	CZ	ARG	687	2.343	58.985	6.234	1.00	25.36	C
ATOM	1531	NH1	ARG	687	1.783	58.118	5.416	1.00	25.62	N
ATOM	1532	NH2	ARG	687	2.342	58.744	7.535	1.00	34.14	N
ATOM	1533	N	ASP	688	−1.044	61.397	3.749	1.00	22.35	N
ATOM	1534	CA	ASP	688	−1.603	60.649	2.632	1.00	20.59	C
ATOM	1535	C	ASP	688	−2.651	61.418	1.872	1.00	23.19	C
ATOM	1536	O	ASP	688	−2.732	61.326	0.653	1.00	23.82	O
ATOM	1537	CB	ASP	688	−2.128	59.301	3.096	1.00	21.54	C
ATOM	1538	CG	ASP	688	−1.008	58.365	3.518	1.00	24.25	C
ATOM	1539	OD1	ASP	688	0.172	58.697	3.266	1.00	31.52	O
ATOM	1540	OD2	ASP	688	−1.287	57.289	4.083	1.00	26.17	O

TABLE 8-continued

Atomic Coordinates for Residues of a Crystal of murine VWF-A1 (SEQ ID NO: 10).

ATOM	1541	N	GLU	689	−3.395	62.253	2.575	1.00	23.99	N
ATOM	1542	CA	GLU	689	−4.419	63.045	1.927	1.00	26.49	C
ATOM	1543	C	GLU	689	−3.796	64.142	1.054	1.00	25.62	C
ATOM	1544	O	GLU	689	−4.376	64.536	0.034	1.00	26.10	O
ATOM	1545	CB	GLU	689	−5.334	63.663	2.981	1.00	34.19	C
ATOM	1546	CG	GLU	689	−6.456	64.543	2.429	1.00	36.12	C
ATOM	1547	CD	GLU	689	−7.358	65.061	3.527	1.00	42.19	C
ATOM	1548	OE1	GLU	689	−7.532	64.327	4.532	1.00	50.63	O
ATOM	1549	OE2	GLU	689	−7.895	66.189	3.385	1.00	42.61	O
ATOM	1550	N	ILE	690	−2.645	64.665	1.478	1.00	21.08	N
ATOM	1551	CA	ILE	690	−1.951	65.705	0.726	1.00	19.71	C
ATOM	1552	C	ILE	690	−1.312	65.124	−0.514	1.00	20.60	C
ATOM	1553	O	ILE	690	−1.368	65.722	−1.567	1.00	23.29	O
ATOM	1554	CB	ILE	690	−0.881	66.388	1.572	1.00	20.01	C
ATOM	1555	CG1	ILE	690	−1.550	67.130	2.737	1.00	18.80	C
ATOM	1556	CG2	ILE	690	−0.054	67.322	0.713	1.00	19.33	C
ATOM	1557	CD1	ILE	690	−0.610	67.889	3.623	1.00	13.89	C
ATOM	1558	N	VAL	691	−0.694	63.958	−0.394	1.00	22.47	N
ATOM	1559	CA	VAL	691	−0.083	63.301	−1.543	1.00	21.73	C
ATOM	1560	C	VAL	691	−1.144	62.902	−2.579	1.00	20.40	C
ATOM	1561	O	VAL	691	−0.923	63.076	−3.767	1.00	22.98	O
ATOM	1562	CB	VAL	691	0.750	62.083	−1.110	1.00	22.75	C
ATOM	1563	CG1	VAL	691	1.147	61.263	−2.296	1.00	29.43	C
ATOM	1564	CG2	VAL	691	1.988	62.555	−0.431	1.00	23.51	C
ATOM	1565	N	SER	692	−2.296	62.407	−2.139	1.00	17.46	N
ATOM	1566	CA	SER	692	−3.371	62.031	−3.054	1.00	15.69	C
ATOM	1567	C	SER	692	−3.894	63.216	−3.856	1.00	16.29	C
ATOM	1568	O	SER	692	−4.106	63.117	−5.060	1.00	19.86	O
ATOM	1569	CB	SER	692	−4.532	61.435	−2.283	1.00	15.65	C
ATOM	1570	OG	SER	692	−4.134	60.230	−1.674	1.00	28.15	O
ATOM	1571	N	TYR	693	−4.129	64.319	−3.168	1.00	16.74	N
ATOM	1572	CA	TYR	693	−4.632	65.536	−3.768	1.00	15.99	C
ATOM	1573	C	TYR	693	−3.654	65.960	−4.833	1.00	18.79	C
ATOM	1574	O	TYR	693	−3.983	66.084	−6.006	1.00	21.36	O
ATOM	1575	CB	TYR	693	−4.725	66.619	−2.702	1.00	11.55	C
ATOM	1576	CG	TYR	693	−5.078	67.969	−3.259	1.00	19.55	C
ATOM	1577	CD1	TYR	693	−6.374	68.238	−3.748	1.00	20.06	C
ATOM	1578	CD2	TYR	693	−4.102	68.960	−3.394	1.00	13.67	C
ATOM	1579	CE1	TYR	693	−6.665	69.458	−4.365	1.00	14.61	C
ATOM	1580	CE2	TYR	693	−4.394	70.165	−4.002	1.00	14.06	C
ATOM	1581	CZ	TYR	693	−5.664	70.402	−4.489	1.00	14.63	C
ATOM	1582	OH	TYR	693	−5.911	71.592	−5.123	1.00	17.27	O
ATOM	1583	N	LEU	694	−2.416	66.070	−4.401	1.00	21.64	N
ATOM	1584	CA	LEU	694	−1.287	66.480	−5.216	1.00	22.01	C
ATOM	1585	C	LEU	694	−1.001	65.549	−6.414	1.00	24.72	C
ATOM	1586	O	LEU	694	−0.819	66.007	−7.560	1.00	21.88	O
ATOM	1587	CB	LEU	694	−0.131	66.631	−4.229	1.00	19.20	C
ATOM	1588	CG	LEU	694	1.365	66.561	−4.388	1.00	23.50	C
ATOM	1589	CD1	LEU	694	1.939	67.326	−3.227	1.00	20.23	C
ATOM	1590	CD2	LEU	694	1.840	65.126	−4.387	1.00	21.48	C
ATOM	1591	N	CYS	695	−1.033	64.243	−6.178	1.00	24.01	N
ATOM	1592	CA	CYS	695	−0.805	63.264	−7.230	1.00	23.59	C
ATOM	1593	C	CYS	695	−1.913	63.363	−8.266	1.00	23.92	C
ATOM	1594	O	CYS	695	−1.662	63.443	−9.476	1.00	20.77	O
ATOM	1595	CB	CYS	695	−0.785	61.851	−6.639	1.00	21.84	C
ATOM	1596	SG	CYS	695	−0.122	60.600	−7.770	1.00	25.31	S
ATOM	1597	N	ASP	696	−3.141	63.401	−7.775	1.00	25.04	N
ATOM	1598	CA	ASP	696	−4.312	63.463	−8.634	1.00	30.16	C
ATOM	1599	C	ASP	696	−4.295	64.617	−9.630	1.00	29.12	C
ATOM	1600	O	ASP	696	−4.933	64.557	−10.678	1.00	33.49	O
ATOM	1601	CB	ASP	696	−5.569	63.536	−7.775	1.00	33.29	C
ATOM	1602	CG	ASP	696	−6.827	63.645	−8.598	1.00	40.85	C
ATOM	1603	OD1	ASP	696	−7.215	62.641	−9.244	1.00	44.44	O
ATOM	1604	OD2	ASP	696	−7.416	64.749	−8.613	1.00	45.63	O
ATOM	1605	N	LEU	697	−3.544	65.655	−9.296	1.00	27.97	N
ATOM	1606	CA	LEU	697	−3.421	66.863	−10.108	1.00	23.65	C
ATOM	1607	C	LEU	697	−2.498	66.673	−11.297	1.00	21.19	C
ATOM	1608	O	LEU	697	−2.676	67.307	−12.337	1.00	23.84	O
ATOM	1609	CB	LEU	697	−2.863	67.990	−9.227	1.00	19.78	C
ATOM	1610	CG	LEU	697	−3.679	69.216	−8.812	1.00	18.30	C
ATOM	1611	CD1	LEU	697	−5.186	68.949	−8.767	1.00	12.42	C
ATOM	1612	CD2	LEU	697	−3.128	69.715	−7.478	1.00	11.15	C
ATOM	1613	N	ALA	698	−1.467	65.864	−11.106	1.00	20.21	N

TABLE 8-continued

Atomic Coordinates for Residues of a Crystal of murine VWF-A1 (SEQ ID NO: 10).

ATOM	1614	CA	ALA	698	−0.472	65.597	−12.128	1.00	18.13	C
ATOM	1615	C	ALA	698	−0.972	64.732	−13.286	1.00	18.60	C
ATOM	1616	O	ALA	698	−1.904	63.935	−13.128	1.00	20.41	O
ATOM	1617	CB	ALA	698	0.746	64.965	−11.481	1.00	16.85	C
ATOM	1618	N	PRO	699	−0.362	64.887	−14.479	1.00	15.84	N
ATOM	1619	CA	PRO	699	−0.802	64.081	−15.622	1.00	14.86	C
ATOM	1620	C	PRO	699	−0.313	62.649	−15.472	1.00	17.66	C
ATOM	1621	O	PRO	699	0.687	62.397	−14.808	1.00	18.33	O
ATOM	1622	CB	PRO	699	−0.131	64.763	−16.817	1.00	10.16	C
ATOM	1623	CG	PRO	699	0.224	66.151	−16.293	1.00	11.52	C
ATOM	1624	CD	PRO	699	0.636	65.888	−14.889	1.00	12.64	C
ATOM	1625	N	GLU	700	−1.029	61.702	−16.058	1.00	18.23	N
ATOM	1626	CA	GLU	700	−0.585	60.329	−15.996	1.00	21.35	C
ATOM	1627	C	GLU	700	0.318	60.141	−17.171	1.00	24.65	C
ATOM	1628	O	GLU	700	0.213	60.873	−18.137	1.00	29.66	O
ATOM	1629	CB	GLU	700	−1.730	59.377	−16.176	1.00	19.18	C
ATOM	1630	CG	GLU	700	−2.640	59.327	−15.036	1.00	27.76	C
ATOM	1631	CD	GLU	700	−3.623	58.221	−15.210	1.00	30.98	C
ATOM	1632	OE1	GLU	700	−3.184	57.061	−15.339	1.00	35.58	O
ATOM	1633	OE2	GLU	700	−4.833	58.511	−15.269	1.00	39.02	O
ATOM	1634	N	ALA	701	1.223	59.173	−17.083	1.00	33.25	N
ATOM	1635	CA	ALA	701	2.109	58.864	−18.203	1.00	34.46	C
ATOM	1636	C	ALA	701	1.251	58.146	−19.254	1.00	36.32	C
ATOM	1637	O	ALA	701	0.277	57.456	−18.916	1.00	30.64	O
ATOM	1638	CB	ALA	701	3.251	57.962	−17.739	1.00	32.72	C
ATOM	1639	N	PRO	702	1.537	58.370	−20.545	1.00	42.71	N
ATOM	1640	CA	PRO	702	0.790	57.732	−21.634	1.00	47.12	C
ATOM	1641	C	PRO	702	1.089	56.237	−21.591	1.00	49.56	C
ATOM	1642	O	PRO	702	2.244	55.851	−21.342	1.00	48.33	O
ATOM	1643	CB	PRO	702	1.402	58.353	−22.886	1.00	45.52	C
ATOM	1644	CG	PRO	702	1.918	59.687	−22.387	1.00	48.78	C
ATOM	1645	CD	PRO	702	2.530	59.322	−21.073	1.00	44.35	C
ATOM	1646	N	PRO	703	0.080	55.383	−21.849	1.00	51.95	N
ATOM	1647	CA	PRO	703	0.189	53.922	−21.841	1.00	54.88	C
ATOM	1648	C	PRO	703	1.480	53.458	−22.521	1.00	59.06	C
ATOM	1649	O	PRO	703	1.819	53.929	−23.616	1.00	56.81	O
ATOM	1650	CB	PRO	703	−1.057	53.477	−22.606	1.00	53.66	C
ATOM	1651	CG	PRO	703	−2.041	54.516	−22.235	1.00	53.29	C
ATOM	1652	CD	PRO	703	−1.240	55.790	−22.379	1.00	53.11	C
ATOM	1653	N	PRO	704	2.271	52.622	−21.816	1.00	64.17	N
ATOM	1654	CA	PRO	704	3.533	52.117	−22.370	1.00	66.20	C
ATOM	1655	C	PRO	704	3.250	51.526	−23.741	1.00	67.33	C
ATOM	1656	O	PRO	704	2.480	50.565	−23.877	1.00	66.63	O
ATOM	1657	CB	PRO	704	3.946	51.050	−21.356	1.00	66.86	C
ATOM	1658	CG	PRO	704	3.457	51.639	−20.067	1.00	65.74	C
ATOM	1659	CD	PRO	704	2.065	52.108	−20.449	1.00	64.51	C
ATOM	1660	N	THR	705	3.885	52.118	−24.741	1.00	67.88	N
ATOM	1661	CA	THR	705	3.712	51.735	−26.130	1.00	68.43	C
ATOM	1662	C	THR	705	5.009	51.114	−26.688	1.00	68.19	C
ATOM	1663	O	THR	705	4.932	50.046	−27.340	1.00	66.67	O
ATOM	1664	CB	THR	705	3.231	52.998	−26.933	1.00	69.60	C
ATOM	1665	OG1	THR	705	2.672	52.619	−28.198	1.00	70.05	O
ATOM	1666	CG2	THR	705	4.357	54.013	−27.124	1.00	68.20	C
ATOM	1667	OXT	THR	705	6.102	51.650	−26.404	1.00	67.96	O

[0319] To demonstrate the feasibility of identifying potential small molecule inhibitors in silico, computational modeling software was utilized in conjunction with high-resolution crystal structure results to screen databases for existing compounds that would bind to the A1 domain where it interfaces with botrocetin (exogenous ligand binding site). Several small molecules predicted to bind with sub-micromolar IC_{50}s (concentration of drug required to inhibit the activity by 50%) and that could also severely disrupt binding of this snake venom protein were identified. Thus, potential candidate small molecules can be identified that may interfere with the interaction between GPIb alpha and the A1 domain of VWF.

[0320] Screening small molecule library for inhibitors. Although the use of computational modeling is a state-of-the-art method for identifying lead compounds, it is not without its limitations. Thus, we will also screen an actual library of 20,000 small molecules manufactured by the Chembridge Corporation (San Diego, Calif.). The library consists of hand-crafted drug-like organic molecules with molecular weights in a range of 25-550, which are soluble in DMSO at concentrations ranging from 10-20 mM. The structure and purity (>95%) of these compounds have been validated by NMR. The library is formatted in a 96 well plate for high throughput screening using instrumentation made available through the OCCC (under supervision of the Landry laboratory) and includes a robot plate reader (FLexStation II 384, Molecular Devices, Sunnyvale, Calif.), an 8-tip robotic pipettor (Multi-

probe II Plus, Perkin Elmer, Shelton, Conn.), a 96-tip robotic pipettor (Mintrak, Perkin Elmer), and an automated 96 well plate washer (Perkin Elmer).

[0321] An ELISA based system will be used to screen for compounds that may inhibit the interaction between GPIb alpha and the VWF-A1 domain. Enzyme-Linked Immunosorbent Assay (ELISA) methods are immunoassay techniques used for detection or quantification of a substance. An example of this assay is demonstrated in FIG. **45**A, where an antibody conjugated with horseradish peroxidase (HRP) was used to identify the presence of VWF. Depending on the substrate added, HRP enzyme activity can be detected by either a change in color (chromogenic product) or fluorescence (most sensitive indicator). Schematic representation of the proposed assay system to be used for screening is shown in FIG. **45**A.

[0322] Assay system: Recombinant GPIb alpha and VWF-A1 proteins will be generated and purified as described in the attached articles, with the latter containing a 6×His tag. Purified GPIb alpha will be absorbed overnight (4° C.) to PROBIND polystyrene 96-well assay plates (Falcon) at 10 μg/ml per well. Plates will be washed and non-specific binding sites blocked by the addition of TENTC buffer (50 mM Tris, 1 mM EDTA, 0.15 M NaCl, 0.2% casein, 0.05% Tween 20, pH 8.0) for 1 hour at room temperature. Subsequently, plates will be washed with and resuspended in TBS buffer (50 mM Tris, 150 mM NaCl, pH 8.0) and 1 test compound per well added at a final concentration of 10 μM (final DMSO concentration 0.5%). After 30 min, recombinant His tagged VWF-A1 protein will be added at a 1:1 Molar ratio to that of GPIb alpha and left to incubate for 1 hour before washing with TBS buffer. VWF-A1 bound to surface-immobilized GPIb alpha will be determined by the addition of HRP-conjugated anti-His tag antibody and the A1-antibody conjugate detected by the addition of LumiGlow reagent (KPL, Gaithersburg, Md.). The resulting fluorescence will be quantified by of the number of luminescence emissions per second using a FLexStation II 384 plate reader. A sample will be considered positive when the luminescence (in counts per second) is more than 2 standard deviations above the mean value for negative-controls.

[0323] Negative controls: Addition of mAb 6D1 to certain wells to prevent VWF-A1 binding to GPIb alpha or no addition of VWF-A1 protein (FIG. **45**B). In either case, no significant fluorescence should be detected. Once compounds of interest have been identified, solubility of these molecules will be confirmed to rule out precipitation as the etiology for blocking interactions between GPIb alpha and VWF-A1. In addition, a dose effect curve will also be generated (1 nM to 100 μM) to obtain preliminary information regarding the IC50 of the inhibitor. Lead molecules will then be tested for their ability to limit human platelet interactions with plasma VWF in aggregometry and flow chamber assays as described in preliminary results. Ultimately, the most promising compound will be tested in our humanized mouse model of thrombosis.

Example 7

Effect of Plavix or ReoPro on Human Platelet-Induced Hemostasis in Homozygous $VWF^{1326R>H}$ Mice

[0324] To demonstrate the feasibility of our $VWF^{1326R>H}$ mice to identify anti-thrombotic drugs capable of perturbing human platelet function in vivo, we tested the ability of 2 FDA approved drugs, Plavix and ReoPro, to prevent human platelet-induced hemostasis. Plavix is the second most commonly used anti-thrombotic drug that targets one of the ADP receptors (P2Y12) on platelets, causing irreversible inhibition (Hankey et al. *Med. J. Aust.* 2003; 178:568). ADP is a potent mediator of platelet activation and aggregate formation, and thus considerable effort and funds have been devoted to inhibiting this activation pathway in platelets. Clopidogrel was approved by the FDA in 1997 for clinical use and was found to be of benefit in the secondary prevention of major vascular events in patients with a history of cerebrovascular and coronary artery diseases and major cardiac events post coronary artery stent placement (Gachet et al. *Semin. Thromb. Hemost.* 2005; 31:162). Disadvantages of this drug are: 1) It must be metabolized in the liver to generate an active metabolite, thus limiting its effectiveness in acute settings, and 2) irreversible inhibition that results in a marked prolongation of bleeding time. Clopidogrel has been shown to reduce thrombus size and delay its formation in mice with a maximal effective dose of 50 mg/kg given the day before and 2 hours prior to experimentation. Homozygous $VWF^{1326R>H}$ mice that received this dosing schema, were unable to produce a hemostatic clot when administered human platelets in contrast to homozygous $VWF^{1326R>H}$ mice that received saline in lieu of drug.

[0325] As Plavix can also block the function of the ADP receptor on murine platelets (see FIG. **46**A), we also tested the ability of ReoPro to prevent the formation of a hemostatic plug in homozygous $VWF^{1326R>H}$ mice. ReoPro is a Fab fragment of a human-murine chimeric monoclonal antibody that blocks fibrinogen binding to the platelet integrin receptor αIIbβ3, thus limiting thrombus growth (Bennett, J. S. Novel platelet inhibitors. *Annu. Rev. Med.* 52, 161-184 (2001)). It is currently approved for short-term treatment of patients with acute coronary syndrome that require interventional catheterization. It is administered by intravenously bolus (0.25 mg/kg), followed by an infusion of 0.125 μg/kg/min. This results in >80% αIIbβ3 occupancy, and disrupts platelet function for 24-36 h. It does not bind or disrupt the function of murine αIIbβ3. Administration of ReoPro to homozygous $VWF^{1326R>H}$ mice 5 minutes after the infusion of human platelets, prevented the formation of a hemostatic plug (mean bleeding time 579 sec) (FIG. **46**B). By contrast, animals that received a non-function blocking antibody to human αIIbβ3 were able to form a hemostatic plug (mean bleeding 175 sec).

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Example 8

Determining the Efficacy of Anti-Platelet Drugs Administered to Patients by Studying the Ability of Platelets Harvested from Patients on Therapies in the $VWF^{1326R>H}$ Mouse

[0397] Intravital microscopic study was carried out to evaluate the ability of the $VWF^{1326R>H}$ mouse to determine the efficacy of anti-platelet therapies given to patients at risk or with active cardiovascular disease. The typical prophylactic dose of aspirin (ASA) of 81 mg did not prevent laser-injury induced human platelet thrombus formation in the genetically modified animal while increasing the daily dose to 162 mg was preventative (FIG. **47**). Similarly, platelets administered from a patient on 81 mg of ASA and 75 mg Plavix also prevented thrombus formation.

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    50                  55                  60

Gly Ser His Ala Tyr Ile Gly Leu Lys Asp Arg Lys Arg Pro Ser Glu
65                  70                  75                  80

Leu Arg Arg Ile Ala Ser Gln Val Lys Tyr Ala Gly Ser Gln Val Ala
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Ser Thr Ser Glu Val Leu Lys Tyr Thr Leu Phe Gln Ile Phe Ser Lys
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Ile Asp Arg Pro Glu Ala Ser Arg Ile Ala Leu Leu Leu Met Ala Ser
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Gln Glu Pro Gln Arg Met Ser Arg Asn Phe Val Arg Tyr Val Gln Gly
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Leu Lys Lys Lys Lys Val Ile Val Ile Pro Val Gly Ile Gly Pro His
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Ala Asn Leu Lys Gln Ile Arg Leu Ile Glu Lys Gln Ala Pro Glu Asn
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Glu Phe Glu Val Leu Lys Ala Phe Val Val Gly Met Met Glu Arg Leu
        35                  40                  45

His Ile Ser Gln Lys Arg Ile Arg Val Ala Val Val Glu Tyr His Asp
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Gly Ser Arg Ala Tyr Leu Glu Leu Lys Ala Arg Lys Arg Pro Ser Glu
65                  70                  75                  80

Leu Arg Arg Ile Thr Ser Gln Ile Lys Tyr Thr Gly Ser Gln Val Ala
                85                  90                  95

Ser Thr Ser Glu Val Leu Lys Tyr Thr Leu Phe Gln Ile Phe Gly Lys
            100                 105                 110

Ile Asp Arg Pro Glu Ala Ser His Ile Thr Leu Leu Leu Thr Ala Ser
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Gln Glu Pro Pro Arg Met Ala Arg Asn Leu Val Arg Tyr Val Gln Gly
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Leu Lys Lys Lys Lys Val Ile Val Ile Pro Val Gly Ile Gly Pro His
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Ala Ser Leu Lys Gln Ile Arg Leu Ile Glu Lys Gln Ala Pro Glu Asn
                165                 170                 175

Lys Ala Phe Leu Leu Ser Gly Val Asp Glu Leu Glu Gln Arg Arg Asp
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Ile Asp Arg Pro Glu Ala Ser His Ile Thr Leu Leu Leu Thr Ala Ser
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Cys Gln Lys Arg Ser Phe Ser Ile Ile Gly Asp Phe Gln Asn Gly Lys
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Phe Val Asn Gly Thr Val Thr Gln Gly Asp Gln Arg Val Ser Met Pro
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Asp Cys Gln Asp His Ser Phe Ser Ile Val Ile Glu Thr Val Gln Cys
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Pro Gly Leu His Asn Ser Leu Val Lys Leu Lys His Gly Ala Gly Val
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Ala Met Asp Gly Gln Asp Ile Gln Leu Pro Leu Leu Lys Gly Asp Leu
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Cys Pro Pro Gly Leu Tyr Met Asp Glu Arg Gly Asp Cys Val Pro Lys
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His Glu Asn Arg Cys Val Ala Leu Glu Arg Cys Pro Cys Phe His Gln
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Gly Lys Glu Tyr Ala Pro Gly Glu Thr Val Lys Ile Gly Cys Asn Thr
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Cys Val Cys Arg Asp Arg Lys Trp Asn Cys Thr Asp His Val Cys Asp
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Ala Thr Cys Ser Thr Ile Gly Met Ala His Tyr Leu Thr Phe Asp Gly
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Ser Trp  Lys Val Ser Ser Gln  Cys Ala Asp Thr Arg  Lys Val Pro
    1025                1030                1035

Leu Asp  Ser Ser Pro Ala Thr  Cys His Asn Asn Ile  Met Lys Gln
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Cys Ile  Tyr Asp Thr Cys Ser  Cys Glu Ser Ile Gly  Asp Cys Ala
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Cys Phe  Cys Asp Thr Ile Ala  Ala Tyr Ala His Val  Cys Ala Gln
    1100                1105                1110

His Gly  Lys Val Val Thr Trp  Arg Thr Ala Thr Leu  Cys Pro Gln
    1115                1120                1125

Ser Cys  Glu Glu Arg Asn Leu  Arg Glu Asn Gly Tyr  Glu Cys Glu
    1130                1135                1140

Trp Arg  Tyr Asn Ser Cys Ala  Pro Ala Cys Gln Val  Thr Cys Gln
    1145                1150                1155

His Pro  Glu Pro Leu Ala Cys  Pro Val Gln Cys Val  Glu Gly Cys
    1160                1165                1170

His Ala  His Cys Pro Pro Gly  Lys Ile Leu Asp Glu  Leu Leu Gln
    1175                1180                1185

Thr Cys  Val Asp Pro Glu Asp  Cys Pro Val Cys Glu  Val Ala Gly
    1190                1195                1200

Arg Arg  Phe Ala Ser Gly Lys  Lys Val Thr Leu Asn  Pro Ser Asp
    1205                1210                1215

Pro Glu  His Cys Gln Ile Cys  His Cys Asp Val Val  Asn Leu Thr
    1220                1225                1230

Cys Glu  Ala Cys Gln Glu Pro  Gly Gly Leu Val Val  Pro Pro Thr
    1235                1240                1245

Asp Ala  Pro Val Ser Pro Thr  Thr Leu Tyr Val Glu  Asp Ile Ser
    1250                1255                1260

Glu Pro  Pro Leu His Asp Phe  Tyr Cys Ser Arg Leu  Leu Asp Leu
    1265                1270                1275

Val Phe  Leu Leu Asp Gly Ser  Ser Arg Leu Ser Glu  Ala Glu Phe
    1280                1285                1290

Glu Val  Leu Lys Ala Phe Val  Val Asp Met Met Glu  Arg Leu Arg
    1295                1300                1305

Ile Ser  Gln Lys Trp Val Arg  Val Ala Val Val Glu  Tyr His Asp
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Gly Ser  His Ala Tyr Ile Gly  Leu Lys Asp Arg Lys  Arg Pro Ser
    1325                1330                1335

Glu Leu  Arg Arg Ile Ala Ser  Gln Val Lys Tyr Ala  Gly Ser Gln
    1340                1345                1350

Val Ala  Ser Thr Ser Glu Val  Leu Lys Tyr Thr Leu  Phe Gln Ile
    1355                1360                1365

Phe Ser  Lys Ile Asp Arg Pro  Glu Ala Ser Arg Ile  Ala Leu Leu
    1370                1375                1380

Leu Met  Ala Ser Gln Glu Pro  Gln Arg Met Ser Arg  Asn Phe Val
    1385                1390                1395

Arg Tyr  Val Gln Gly Leu Lys  Lys Lys Lys Val Ile  Val Ile Pro
    1400                1405                1410

Val Gly  Ile Gly Pro His Ala  Asn Leu Lys Gln Ile  Arg Leu Ile
    1415                1420                1425

Glu Lys  Gln Ala Pro Glu Asn  Lys Ala Phe Val Leu  Ser Ser Val
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    1430                1435                1440

Asp Glu  Leu Glu Gln Gln Arg  Asp Glu Ile Val Ser  Tyr Leu Cys
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Asp Leu  Ala Pro Glu Ala Pro  Pro Pro Thr Leu Pro  Pro His Met
    1460                1465                1470

Ala Gln  Val Thr Val Gly Pro  Gly Leu Leu Gly Val  Ser Thr Leu
    1475                1480                1485

Gly Pro  Lys Arg Asn Ser Met  Val Leu Asp Val Ala  Phe Val Leu
    1490                1495                1500

Glu Gly  Ser Asp Lys Ile Gly  Glu Ala Asp Phe Asn  Arg Ser Lys
    1505                1510                1515

Glu Phe  Met Glu Glu Val Ile  Gln Arg Met Asp Val  Gly Gln Asp
    1520                1525                1530

Ser Ile  His Val Thr Val Leu  Gln Tyr Ser Tyr Met  Val Thr Val
    1535                1540                1545

Glu Tyr  Pro Phe Ser Glu Ala  Gln Ser Lys Gly Asp  Ile Leu Gln
    1550                1555                1560

Arg Val  Arg Glu Ile Arg Tyr  Gln Gly Gly Asn Arg  Thr Asn Thr
    1565                1570                1575

Gly Leu  Ala Leu Arg Tyr Leu  Ser Asp His Ser Phe  Leu Val Ser
    1580                1585                1590

Gln Gly  Asp Arg Glu Gln Ala  Pro Asn Leu Val Tyr  Met Val Thr
    1595                1600                1605

Gly Asn  Pro Ala Ser Asp Glu  Ile Lys Arg Leu Pro  Gly Asp Ile
    1610                1615                1620

Gln Val  Val Pro Ile Gly Val  Gly Pro Asn Ala Asn  Val Gln Glu
    1625                1630                1635

Leu Glu  Arg Ile Gly Trp Pro  Asn Ala Pro Ile Leu  Ile Gln Asp
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Phe Glu  Thr Leu Pro Arg Glu  Ala Pro Asp Leu Val  Leu Gln Arg
    1655                1660                1665

Cys Cys  Ser Gly Glu Gly Leu  Gln Ile Pro Thr Leu  Ser Pro Ala
    1670                1675                1680

Pro Asp  Cys Ser Gln Pro Leu  Asp Val Ile Leu Leu  Leu Asp Gly
    1685                1690                1695

Ser Ser  Ser Phe Pro Ala Ser  Tyr Phe Asp Glu Met  Lys Ser Phe
    1700                1705                1710

Ala Lys  Ala Phe Ile Ser Lys  Ala Asn Ile Gly Pro  Arg Leu Thr
    1715                1720                1725

Gln Val  Ser Val Leu Gln Tyr  Gly Ser Ile Thr Thr  Ile Asp Val
    1730                1735                1740

Pro Trp  Asn Val Val Pro Glu  Lys Ala His Leu Leu  Ser Leu Val
    1745                1750                1755

Asp Val  Met Gln Arg Glu Gly  Gly Pro Ser Gln Ile  Gly Asp Ala
    1760                1765                1770

Leu Gly  Phe Ala Val Arg Tyr  Leu Thr Ser Glu Met  His Gly Ala
    1775                1780                1785

Arg Pro  Gly Ala Ser Lys Ala  Val Val Ile Leu Val  Thr Asp Val
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Ser Val  Asp Ser Val Asp Ala  Ala Ala Asp Ala Ala  Arg Ser Asn
    1805                1810                1815
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Arg Val  Thr Val Phe Pro Ile  Gly Ile Gly Asp Arg  Tyr Asp Ala
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Ala Gln  Leu Arg Ile Leu Ala  Gly Pro Ala Gly Asp  Ser Asn Val
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Val Lys  Leu Gln Arg Ile Glu  Asp Leu Pro Thr Met  Val Thr Leu
    1850                1855                1860

Gly Asn  Ser Phe Leu His Lys  Leu Cys Ser Gly Phe  Val Arg Ile
    1865                1870                1875

Cys Met  Asp Glu Asp Gly Asn  Glu Lys Arg Pro Gly  Asp Val Trp
    1880                1885                1890

Thr Leu  Pro Asp Gln Cys His  Thr Val Thr Cys Gln  Pro Asp Gly
    1895                1900                1905

Gln Thr  Leu Leu Lys Ser His  Arg Val Asn Cys Asp  Arg Gly Leu
    1910                1915                1920

Arg Pro  Ser Cys Pro Asn Ser  Gln Ser Pro Val Lys  Val Glu Glu
    1925                1930                1935

Thr Cys  Gly Cys Arg Trp Thr  Cys Pro Cys Val Cys  Thr Gly Ser
    1940                1945                1950

Ser Thr  Arg His Ile Val Thr  Phe Asp Gly Gln Asn  Phe Lys Leu
    1955                1960                1965

Thr Gly  Ser Cys Ser Tyr Val  Leu Phe Gln Asn Lys  Glu Gln Asp
    1970                1975                1980

Leu Glu  Val Ile Leu His Asn  Gly Ala Cys Ser Pro  Gly Ala Arg
    1985                1990                1995

Gln Gly  Cys Met Lys Ser Ile  Glu Val Lys His Ser  Ala Leu Ser
    2000                2005                2010

Val Glu  Leu His Ser Asp Met  Glu Val Thr Val Asn  Gly Arg Leu
    2015                2020                2025

Val Ser  Val Pro Tyr Val Gly  Gly Asn Met Glu Val  Asn Val Tyr
    2030                2035                2040

Gly Ala  Ile Met His Glu Val  Arg Phe Asn His Leu  Gly His Ile
    2045                2050                2055

Phe Thr  Phe Thr Pro Gln Asn  Asn Glu Phe Gln Leu  Gln Leu Ser
    2060                2065                2070

Pro Lys  Thr Phe Ala Ser Lys  Thr Tyr Gly Leu Cys  Gly Ile Cys
    2075                2080                2085

Asp Glu  Asn Gly Ala Asn Asp  Phe Met Leu Arg Asp  Gly Thr Val
    2090                2095                2100

Thr Thr  Asp Trp Lys Thr Leu  Val Gln Glu Trp Thr  Val Gln Arg
    2105                2110                2115

Pro Gly  Gln Thr Cys Gln Pro  Ile Leu Glu Glu Gln  Cys Leu Val
    2120                2125                2130

Pro Asp  Ser Ser His Cys Gln  Val Leu Leu Leu Pro  Leu Phe Ala
    2135                2140                2145

Glu Cys  His Lys Val Leu Ala  Pro Ala Thr Phe Tyr  Ala Ile Cys
    2150                2155                2160

Gln Gln  Asp Ser Cys His Gln  Glu Gln Val Cys Glu  Val Ile Ala
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Ser Tyr  Ala His Leu Cys Arg  Thr Asn Gly Val Cys  Val Asp Trp
    2180                2185                2190
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Tyr Asn  His Cys Glu His Gly  Cys Pro Arg His Cys  Asp Gly Asn
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Val Ser  Ser Cys Gly Asp His  Pro Ser Glu Gly Cys  Phe Cys Pro
    2225                 2230                 2235

Pro Asp  Lys Val Met Leu Glu  Gly Ser Cys Val Pro  Glu Glu Ala
    2240                 2245                 2250

Cys Thr  Gln Cys Ile Gly Glu  Asp Gly Val Gln His  Gln Phe Leu
    2255                 2260                 2265

Glu Ala  Trp Val Pro Asp His  Gln Pro Cys Gln Ile  Cys Thr Cys
    2270                 2275                 2280

Leu Ser  Gly Arg Lys Val Asn  Cys Thr Thr Gln Pro  Cys Pro Thr
    2285                 2290                 2295

Ala Lys  Ala Pro Thr Cys Gly  Leu Cys Glu Val Ala  Arg Leu Arg
    2300                 2305                 2310

Gln Asn  Ala Asp Gln Cys Cys  Pro Glu Tyr Glu Cys  Val Cys Asp
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Pro Val  Ser Cys Asp Leu Pro  Pro Val Pro His Cys  Glu Arg Gly
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Leu Gln  Pro Thr Leu Thr Asn  Pro Gly Glu Cys Arg  Pro Asn Phe
    2345                 2350                 2355

Thr Cys  Ala Cys Arg Lys Glu  Glu Cys Lys Arg Val  Ser Pro Pro
    2360                 2365                 2370

Ser Cys  Pro Pro His Arg Leu  Pro Thr Leu Arg Lys  Thr Gln Cys
    2375                 2380                 2385

Cys Asp  Glu Tyr Glu Cys Ala  Cys Asn Cys Val Asn  Ser Thr Val
    2390                 2395                 2400

Ser Cys  Pro Leu Gly Tyr Leu  Ala Ser Thr Ala Thr  Asn Asp Cys
    2405                 2410                 2415

Gly Cys  Thr Thr Thr Thr Cys  Leu Pro Asp Lys Val  Cys Val His
    2420                 2425                 2430

Arg Ser  Thr Ile Tyr Pro Val  Gly Gln Phe Trp Glu  Glu Gly Cys
    2435                 2440                 2445

Asp Val  Cys Thr Cys Thr Asp  Met Glu Asp Ala Val  Met Gly Leu
    2450                 2455                 2460

Arg Val  Ala Gln Cys Ser Gln  Lys Pro Cys Glu Asp  Ser Cys Arg
    2465                 2470                 2475

Ser Gly  Phe Thr Tyr Val Leu  His Glu Gly Glu Cys  Cys Gly Arg
    2480                 2485                 2490

Cys Leu  Pro Ser Ala Cys Glu  Val Val Thr Gly Ser  Pro Arg Gly
    2495                 2500                 2505

Asp Ser  Gln Ser Ser Trp Lys  Ser Val Gly Ser Gln  Trp Ala Ser
    2510                 2515                 2520

Pro Glu  Asn Pro Cys Leu Ile  Asn Glu Cys Val Arg  Val Lys Glu
    2525                 2530                 2535

Glu Val  Phe Ile Gln Gln Arg  Asn Val Ser Cys Pro  Gln Leu Glu
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Val Pro  Val Cys Pro Ser Gly  Phe Gln Leu Ser Cys  Lys Thr Ser
    2555                 2560                 2565

Ala Cys  Cys Pro Ser Cys Arg  Cys Glu Arg Met Glu  Ala Cys Met
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Leu Asn  Gly Thr Val Ile Gly  Pro Gly Lys Thr Val  Met Ile Asp
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Val Cys  Thr Thr Cys Arg Cys  Met Val Gln Val Gly  Val Ile Ser
    2600                2605                2610

Gly Phe  Lys Leu Glu Cys Arg  Lys Thr Thr Cys Asn  Pro Cys Pro
    2615                2620                2625

Leu Gly  Tyr Lys Glu Glu Asn  Asn Thr Gly Glu Cys  Cys Gly Arg
    2630                2635                2640

Cys Leu  Pro Thr Ala Cys Thr  Ile Gln Leu Arg Gly  Gly Gln Ile
    2645                2650                2655

Met Thr  Leu Lys Arg Asp Glu  Thr Leu Gln Asp Gly  Cys Asp Thr
    2660                2665                2670

His Phe  Cys Lys Val Asn Glu  Arg Gly Glu Tyr Phe  Trp Glu Lys
    2675                2680                2685

Arg Val  Thr Gly Cys Pro Pro  Phe Asp Glu His Lys  Cys Leu Ala
    2690                2695                2700

Glu Gly  Gly Lys Ile Met Lys  Ile Pro Gly Thr Cys  Cys Asp Thr
    2705                2710                2715

Cys Glu  Glu Pro Glu Cys Asn  Asp Ile Thr Ala Arg  Leu Gln Tyr
    2720                2725                2730

Val Lys  Val Gly Ser Cys Lys  Ser Glu Val Glu Val  Asp Ile His
    2735                2740                2745

Tyr Cys  Gln Gly Lys Cys Ala  Ser Lys Ala Met Tyr  Ser Ile Asp
    2750                2755                2760

Ile Asn  Asp Val Gln Asp Gln  Cys Ser Cys Cys Ser  Pro Thr Arg
    2765                2770                2775

Thr Glu  Pro Met Gln Val Ala  Leu His Cys Thr Asn  Gly Ser Val
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Val Tyr  His Glu Val Leu Asn  Ala Met Glu Cys Lys  Cys Ser Pro
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Arg Lys  Cys Ser Lys
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<211> LENGTH: 2813
<212> TYPE: PRT
<213> ORGANISM: Mus musculus

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Trp Pro Gly Thr Leu Cys Thr Glu Lys Pro Arg Asp Arg Pro Ser Thr
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Ala Arg Cys Ser Leu Phe Gly Asp Asp Phe Ile Asn Thr Phe Asp Glu
35 40 45

Thr Met Tyr Ser Phe Ala Gly Gly Cys Ser Tyr Leu Leu Ala Gly Asp
50 55 60

Cys Gln Lys Arg Ser Phe Ser Ile Leu Gly Asn Phe Gln Asp Gly Lys
65 70 75 80

Arg Met Ser Leu Ser Val Tyr Leu Gly Glu Phe Phe Asp Ile His Leu

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               85                  90                  95

Phe Ala Asn Gly Thr Val Thr Gln Gly Asp Gln Ser Ile Ser Met Pro
            100                 105                 110

Tyr Ala Ser Gln Gly Leu Tyr Leu Glu Arg Glu Ala Gly Tyr Tyr Lys
        115                 120                 125

Leu Ser Ser Glu Thr Phe Gly Phe Ala Ala Arg Ile Asp Gly Asn Gly
    130                 135                 140

Asn Phe Gln Val Leu Met Ser Asp Arg His Phe Asn Lys Thr Cys Gly
145                 150                 155                 160

Leu Cys Gly Asp Phe Asn Ile Phe Ala Glu Asp Asp Phe Arg Thr Gln
                165                 170                 175

Glu Gly Thr Leu Thr Ser Asp Pro Tyr Asp Phe Ala Asn Ser Trp Ala
            180                 185                 190

Leu Ser Ser Glu Glu Gln Arg Cys Lys Arg Ala Ser Pro Pro Ser Arg
        195                 200                 205

Asn Cys Glu Ser Ser Ser Gly Asp Met His Gln Ala Met Trp Glu Gln
    210                 215                 220

Cys Gln Leu Leu Lys Thr Ala Ser Val Phe Ala Arg Cys His Pro Leu
225                 230                 235                 240

Val Asp Pro Glu Ser Phe Val Ala Leu Cys Glu Lys Ile Leu Cys Thr
                245                 250                 255

Cys Ala Thr Gly Pro Glu Cys Ala Cys Pro Val Leu Leu Glu Tyr Ala
            260                 265                 270

Arg Thr Cys Ala Gln Glu Gly Met Val Leu Tyr Gly Trp Thr Asp His
        275                 280                 285

Ser Ala Cys Arg Pro Ala Cys Pro Ala Gly Met Glu Tyr Lys Glu Cys
    290                 295                 300

Val Ser Pro Cys Pro Arg Thr Cys Gln Ser Leu Ser Ile Asn Glu Val
305                 310                 315                 320

Cys Gln Gln Gln Cys Val Asp Gly Cys Ser Cys Pro Glu Gly Glu Leu
                325                 330                 335

Leu Asp Glu Asp Arg Cys Val Gln Ser Ser Asp Cys Pro Cys Val His
            340                 345                 350

Ala Gly Lys Arg Tyr Pro Pro Gly Thr Ser Leu Ser Gln Asp Cys Asn
        355                 360                 365

Thr Cys Ile Cys Arg Asn Ser Leu Trp Ile Cys Ser Asn Glu Glu Cys
    370                 375                 380

Pro Gly Glu Cys Leu Val Thr Gly Gln Ser His Phe Lys Ser Phe Asp
385                 390                 395                 400

Asn Arg Tyr Phe Thr Phe Ser Gly Ile Cys Gln Tyr Leu Leu Ala Arg
                405                 410                 415

Asp Cys Glu Asp His Thr Phe Ser Ile Val Ile Glu Thr Met Gln Cys
            420                 425                 430

Ala Asp Asp Pro Asp Ala Val Cys Thr Arg Ser Val Ser Val Arg Leu
        435                 440                 445

Ser Ala Leu His Asn Ser Leu Val Lys Leu Lys His Gly Gly Ala Val
    450                 455                 460

Gly Ile Asp Gly Gln Asp Val Gln Leu Pro Phe Leu Gln Gly Asp Leu
465                 470                 475                 480

Arg Ile Gln His Thr Val Met Ala Ser Val Arg Leu Ser Tyr Ala Glu
                485                 490                 495
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Asp Leu Gln Met Asp Trp Asp Gly Arg Gly Arg Leu Leu Val Lys Leu
            500                 505                 510

Ser Pro Val Tyr Ser Gly Lys Thr Cys Gly Leu Cys Gly Asn Tyr Asn
        515                 520                 525

Gly Asn Lys Gly Asp Asp Phe Leu Thr Pro Ala Gly Leu Val Glu Pro
    530                 535                 540

Leu Val Val Asp Phe Gly Asn Ala Trp Lys Leu Gln Gly Asp Cys Ser
545                 550                 555                 560

Asp Leu Arg Arg Gln His Ser Asp Pro Cys Ser Leu Asn Pro Arg Leu
                565                 570                 575

Thr Arg Phe Ala Glu Glu Ala Cys Ala Leu Leu Thr Ser Ser Lys Phe
            580                 585                 590

Glu Ala Cys His His Ala Val Ser Pro Leu Pro Tyr Leu Gln Asn Cys
        595                 600                 605

Arg Tyr Asp Val Cys Ser Cys Ser Asp Ser Arg Asp Cys Leu Cys Asn
    610                 615                 620

Ala Val Ala Asn Tyr Ala Ala Glu Cys Ala Arg Lys Gly Val His Ile
625                 630                 635                 640

Gly Trp Arg Glu Pro Gly Phe Cys Ala Leu Gly Cys Pro Gln Gly Gln
                645                 650                 655

Val Tyr Leu Gln Cys Gly Asn Ser Cys Asn Leu Thr Cys Arg Ser Leu
            660                 665                 670

Ser Leu Pro Asp Glu Glu Cys Ser Glu Val Cys Leu Glu Gly Cys Tyr
        675                 680                 685

Cys Pro Pro Gly Leu Tyr Gln Asp Glu Arg Gly Asp Cys Val Pro Lys
    690                 695                 700

Ala Gln Cys Pro Cys Tyr Tyr Asp Gly Glu Leu Phe Gln Pro Ala Asp
705                 710                 715                 720

Ile Phe Ser Asp His His Thr Met Cys Tyr Cys Glu Asp Gly Phe Met
                725                 730                 735

His Cys Thr Thr Ser Gly Thr Leu Gly Ser Leu Leu Pro Asp Thr Val
            740                 745                 750

Leu Ser Ser Pro Leu Ser His Arg Ser Lys Arg Ser Leu Ser Cys Arg
        755                 760                 765

Pro Pro Met Val Lys Leu Val Cys Pro Ala Asp Asn Pro Arg Ala Gln
    770                 775                 780

Gly Leu Glu Cys Ala Lys Thr Cys Gln Asn Tyr Asp Leu Glu Cys Met
785                 790                 795                 800

Ser Leu Gly Cys Val Ser Gly Cys Leu Cys Pro Pro Gly Met Val Arg
                805                 810                 815

His Glu Asn Lys Cys Val Ala Leu Glu Arg Cys Pro Cys Phe His Gln
            820                 825                 830

Gly Ala Glu Tyr Ala Pro Gly Asp Thr Val Lys Ile Gly Cys Asn Thr
        835                 840                 845

Cys Val Cys Arg Glu Arg Lys Trp Asn Cys Thr Asn His Val Cys Asp
    850                 855                 860

Ala Thr Cys Ser Ala Ile Gly Met Ala His Tyr Leu Thr Phe Asp Gly
865                 870                 875                 880

Leu Lys Tyr Leu Phe Pro Gly Glu Cys Gln Tyr Val Leu Val Gln Asp
                885                 890                 895
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Tyr Cys Gly Ser Asn Pro Gly Thr Phe Gln Ile Leu Val Gly Asn Glu
           900                 905                 910

Gly Cys Ser Tyr Pro Ser Val Lys Cys Arg Lys Arg Val Thr Ile Leu
        915                 920                 925

Val Asp Gly Gly Glu Leu Glu Leu Phe Asp Gly Glu Val Asn Val Lys
    930                 935                 940

Arg Pro Leu Arg Asp Glu Ser His Phe Glu Val Val Glu Ser Gly Arg
945                 950                 955                 960

Tyr Val Ile Leu Leu Leu Gly Gln Ala Leu Ser Val Val Trp Asp His
               965                 970                 975

His Leu Ser Ile Ser Val Val Leu Lys His Thr Tyr Gln Glu Gln Val
           980                 985                 990

Cys Gly Leu Cys Gly Asn Phe Asp  Gly Ile Gln Asn Asn  Asp Phe Thr
        995                 1000                1005

Thr Ser  Ser Leu Gln Val Glu  Glu Asp Pro Val Asn  Phe Gly Asn
    1010                1015                1020

Ser Trp  Lys Val Ser Ser Gln  Cys Ala Asp Thr Arg  Lys Leu Ser
    1025                1030                1035

Leu Asp  Val Ser Pro Ala Thr  Cys His Asn Asn Ile  Met Lys Gln
    1040                1045                1050

Thr Met  Val Asp Ser Ala Cys  Arg Ile Leu Thr Ser  Asp Val Phe
    1055                1060                1065

Gln Gly  Cys Asn Arg Leu Val  Asp Pro Glu Pro Tyr  Leu Asp Ile
    1070                1075                1080

Cys Ile  Tyr Asp Thr Cys Ser  Cys Glu Ser Ile Gly  Asp Cys Ala
    1085                1090                1095

Cys Phe  Cys Asp Thr Ile Ala  Ala Tyr Ala His Val  Cys Ala Gln
    1100                1105                1110

His Gly  Gln Val Val Ala Trp  Arg Thr Pro Thr Leu  Cys Pro Gln
    1115                1120                1125

Ser Cys  Glu Glu Lys Asn Val  Arg Glu Asn Gly Tyr  Glu Cys Glu
    1130                1135                1140

Trp Arg  Tyr Asn Ser Cys Ala  Pro Ala Cys Pro Val  Thr Cys Gln
    1145                1150                1155

His Pro  Glu Pro Leu Ala Cys  Pro Val Gln Cys Val  Glu Gly Cys
    1160                1165                1170

His Ala  His Cys Pro Pro Gly  Arg Ile Leu Asp Glu  Leu Leu Gln
    1175                1180                1185

Thr Cys  Val Asp Pro Gln Asp  Cys Pro Val Cys Glu  Val Ala Gly
    1190                1195                1200

Arg Arg  Leu Ala Pro Gly Lys  Lys Ile Thr Leu Ser  Pro Asp Asp
    1205                1210                1215

Pro Ala  His Cys Gln Asn Cys  His Cys Asp Gly Val  Asn Leu Thr
    1220                1225                1230

Cys Glu  Ala Cys Gln Glu Pro  Gly Gly Leu Val Ala  Pro Pro Thr
    1235                1240                1245

Asp Ala  Pro Val Ser Ser Thr  Thr Pro Tyr Val Glu  Asp Thr Pro
    1250                1255                1260

Glu Pro  Pro Leu His Asn Phe  Tyr Cys Ser Lys Leu  Leu Asp Leu
    1265                1270                1275

Val Phe  Leu Leu Asp Gly Ser  Ser Met Leu Ser Glu  Ala Glu Phe
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    1280                1285                1290

Glu Val  Leu Lys Ala Phe Val  Val Gly Met Met Glu  Arg Leu His
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Ile Ser  Gln Lys Arg Ile Arg  Val Ala Val Val Glu  Tyr His Asp
    1310                1315                1320

Gly Ser  Arg Ala Tyr Leu Glu  Leu Lys Ala Arg Lys  Arg Pro Ser
    1325                1330                1335

Glu Leu  Arg Arg Ile Thr Ser  Gln Ile Lys Tyr Thr  Gly Ser Gln
    1340                1345                1350

Val Ala  Ser Thr Ser Glu Val  Leu Lys Tyr Thr Leu  Phe Gln Ile
    1355                1360                1365

Phe Gly  Lys Ile Asp Arg Pro  Glu Ala Ser His Ile  Thr Leu Leu
    1370                1375                1380

Leu Thr  Ala Ser Gln Glu Pro  Pro Arg Met Ala Arg  Asn Leu Val
    1385                1390                1395

Arg Tyr  Val Gln Gly Leu Lys  Lys Lys Lys Val Ile  Val Ile Pro
    1400                1405                1410

Val Gly  Ile Gly Pro His Ala  Ser Leu Lys Gln Ile  Arg Leu Ile
    1415                1420                1425

Glu Lys  Gln Ala Pro Glu Asn  Lys Ala Phe Leu Leu  Ser Gly Val
    1430                1435                1440

Asp Glu  Leu Glu Gln Arg Arg  Asp Glu Ile Val Ser  Tyr Leu Cys
    1445                1450                1455

Asp Leu  Ala Pro Glu Ala Pro  Ala Pro Thr Gln Pro  Pro Gln Val
    1460                1465                1470

Ala His  Val Thr Val Ser Pro  Gly Ile Ala Gly Ile  Ser Ser Pro
    1475                1480                1485

Gly Pro  Lys Arg Lys Ser Met  Val Leu Asp Val Val  Phe Val Leu
    1490                1495                1500

Glu Gly  Ser Asp Glu Val Gly  Glu Ala Asn Phe Asn  Lys Ser Lys
    1505                1510                1515

Glu Phe  Val Glu Glu Val Ile  Gln Arg Met Asp Val  Ser Pro Asp
    1520                1525                1530

Ala Thr  Arg Ile Ser Val Leu  Gln Tyr Ser Tyr Thr  Val Thr Met
    1535                1540                1545

Glu Tyr  Ala Phe Asn Gly Ala  Gln Ser Lys Glu Glu  Val Leu Arg
    1550                1555                1560

His Val  Arg Glu Ile Arg Tyr  Gln Gly Gly Asn Arg  Thr Asn Thr
    1565                1570                1575

Gly Gln  Ala Leu Gln Tyr Leu  Ser Glu His Ser Phe  Ser Pro Ser
    1580                1585                1590

Gln Gly  Asp Arg Val Glu Ala  Pro Asn Leu Val Tyr  Met Val Thr
    1595                1600                1605

Gly Asn  Pro Ala Ser Asp Glu  Ile Lys Arg Leu Pro  Gly Asp Ile
    1610                1615                1620

Gln Val  Val Pro Ile Gly Val  Gly Pro His Ala Asn  Met Gln Glu
    1625                1630                1635

Leu Glu  Arg Ile Ser Arg Pro  Ile Ala Pro Ile Phe  Ile Arg Asp
    1640                1645                1650

Phe Glu  Thr Leu Pro Arg Glu  Ala Pro Asp Leu Val  Leu Gln Thr
    1655                1660                1665
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Cys Cys  Ser Lys Glu Gly Leu  Gln Leu Pro Thr Leu  Pro Pro Leu
    1670                1675                1680

Pro Asp  Cys Ser Gln Pro Leu  Asp Val Val Leu Leu  Leu Asp Gly
    1685                1690                1695

Ser Ser  Ser Leu Pro Glu Ser  Ser Phe Asp Lys Met  Lys Ser Phe
    1700                1705                1710

Ala Lys  Ala Phe Ile Ser Lys  Ala Asn Ile Gly Pro  His Leu Thr
    1715                1720                1725

Gln Val  Ser Val Ile Gln Tyr  Gly Ser Ile Asn Thr  Ile Asp Val
    1730                1735                1740

Pro Trp  Asn Val Val Gln Glu  Lys Ala His Leu Gln  Ser Leu Val
    1745                1750                1755

Asp Leu  Met Gln Gln Glu Gly  Gly Pro Ser Gln Ile  Gly Asp Ala
    1760                1765                1770

Leu Ala  Phe Ala Val Arg Tyr  Val Thr Ser Gln Ile  His Gly Ala
    1775                1780                1785

Arg Pro  Gly Ala Ser Lys Ala  Val Val Ile Ile Ile  Met Asp Thr
    1790                1795                1800

Ser Leu  Asp Pro Val Asp Thr  Ala Ala Asp Ala Ala  Arg Ser Asn
    1805                1810                1815

Arg Val  Ala Val Phe Pro Val  Gly Val Gly Asp Arg  Tyr Asp Glu
    1820                1825                1830

Ala Gln  Leu Arg Ile Leu Ala  Gly Pro Gly Ala Ser  Ser Asn Val
    1835                1840                1845

Val Lys  Leu Gln Gln Val Glu  Asp Leu Ser Thr Met  Ala Thr Leu
    1850                1855                1860

Gly Asn  Ser Phe Phe His Lys  Leu Cys Ser Gly Phe  Ser Gly Val
    1865                1870                1875

Cys Val  Asp Glu Asp Gly Asn  Glu Lys Arg Pro Gly  Asp Val Trp
    1880                1885                1890

Thr Leu  Pro Asp Gln Cys His  Thr Val Thr Cys Leu  Ala Asn Gly
    1895                1900                1905

Gln Thr  Leu Leu Gln Ser His  Arg Val Asn Cys Asp  His Gly Pro
    1910                1915                1920

Arg Pro  Ser Cys Ala Asn Ser  Gln Ser Pro Val Arg  Val Glu Glu
    1925                1930                1935

Thr Cys  Gly Cys Arg Trp Thr  Cys Pro Cys Val Cys  Thr Gly Ser
    1940                1945                1950

Ser Thr  Arg His Ile Val Thr  Phe Asp Gly Gln Asn  Phe Lys Leu
    1955                1960                1965

Thr Gly  Ser Cys Ser Tyr Val  Ile Phe Gln Asn Lys  Glu Gln Asp
    1970                1975                1980

Leu Glu  Val Leu Leu His Asn  Gly Ala Cys Ser Pro  Gly Ala Lys
    1985                1990                1995

Gln Ala  Cys Met Lys Ser Ile  Glu Ile Lys His Ala  Gly Val Ser
    2000                2005                2010

Ala Glu  Leu His Ser Asn Met  Glu Met Ala Val Asp  Gly Arg Leu
    2015                2020                2025

Val Leu  Ala Pro Tyr Val Gly  Glu Asn Met Glu Val  Ser Ile Tyr
    2030                2035                2040
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Gly Ala  Ile Met Tyr Glu Val  Arg Phe Thr His Leu  Gly His Ile
    2045                2050                2055

Leu Thr  Tyr Thr Pro Gln Asn  Asn Glu Phe Gln Leu  Gln Leu Ser
    2060                2065                2070

Pro Lys  Thr Phe Ala Ser Lys  Met His Gly Leu Cys  Gly Ile Cys
    2075                2080                2085

Asp Glu  Asn Gly Ala Asn Asp  Phe Thr Leu Arg Asp  Gly Thr Val
    2090                2095                2100

Thr Thr  Asp Trp Lys Arg Leu  Val Gln Glu Trp Thr  Val Gln Gln
    2105                2110                2115

Pro Gly  Tyr Thr Cys Gln Ala  Val Pro Glu Glu Gln  Cys Pro Val
    2120                2125                2130

Ser Asp  Ser Ser His Cys Gln  Val Leu Leu Ser Ala  Ser Phe Ala
    2135                2140                2145

Glu Cys  His Lys Val Ile Ala  Pro Ala Thr Phe His  Thr Ile Cys
    2150                2155                2160

Gln Gln  Asp Ser Cys His Gln  Glu Arg Val Cys Glu  Val Ile Ala
    2165                2170                2175

Ser Tyr  Ala His Leu Cys Arg  Thr Ser Gly Val Cys  Val Asp Trp
    2180                2185                2190

Arg Thr  Thr Asp Phe Cys Ala  Met Ser Cys Pro Pro  Ser Leu Val
    2195                2200                2205

Tyr Asn  His Cys Glu Arg Gly  Cys Pro Arg His Cys  Asp Gly Asn
    2210                2215                2220

Thr Ser  Phe Cys Gly Asp His  Pro Ser Glu Gly Cys  Phe Cys Pro
    2225                2230                2235

Gln His  Gln Val Phe Leu Glu  Gly Ser Cys Val Pro  Glu Glu Ala
    2240                2245                2250

Cys Thr  Gln Cys Val Gly Glu  Asp Gly Val Arg His  Gln Phe Leu
    2255                2260                2265

Glu Thr  Trp Val Pro Asp His  Gln Pro Cys Gln Ile  Cys Met Cys
    2270                2275                2280

Leu Ser  Gly Arg Lys Ile Asn  Cys Thr Ala Gln Pro  Cys Pro Thr
    2285                2290                2295

Ala Arg  Ala Pro Thr Cys Gly  Pro Cys Glu Val Ala  Arg Leu Lys
    2300                2305                2310

Gln Ser  Thr Asn Leu Cys Cys  Pro Glu Tyr Glu Cys  Val Cys Asp
    2315                2320                2325

Leu Phe  Asn Cys Asn Leu Pro  Pro Val Pro Pro Cys  Glu Gly Gly
    2330                2335                2340

Leu Gln  Pro Thr Leu Thr Asn  Pro Gly Glu Cys Arg  Pro Thr Phe
    2345                2350                2355

Thr Cys  Ala Cys Arg Lys Glu  Glu Cys Lys Arg Val  Ser Pro Pro
    2360                2365                2370

Ser Cys  Pro Pro His Arg Thr  Pro Thr Leu Arg Lys  Thr Gln Cys
    2375                2380                2385

Cys Asp  Glu Tyr Glu Cys Ala  Cys Ser Cys Val Asn  Ser Thr Leu
    2390                2395                2400

Ser Cys  Pro Leu Gly Tyr Leu  Ala Ser Ala Thr Thr  Asn Asp Cys
    2405                2410                2415

Gly Cys  Thr Thr Thr Thr Cys  Leu Pro Asp Lys Val  Cys Val His
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Arg Gly  Thr Val Tyr Pro Val  Gly Gln Phe Trp Glu  Glu Gly Cys
    2435                2440                2445

Asp Thr  Cys Thr Cys Thr Asp  Met Glu Asp Thr Val  Val Gly Leu
    2450                2455                2460

Arg Val  Val Gln Cys Ser Gln  Arg Pro Cys Glu Asp  Ser Cys Gln
    2465                2470                2475

Pro Gly  Phe Ser Tyr Val Leu  His Glu Gly Glu Cys  Cys Gly Arg
    2480                2485                2490

Cys Leu  Pro Ser Ala Cys Lys  Val Val Ala Gly Ser  Leu Arg Gly
    2495                2500                2505

Asp Ser  His Ser Ser Trp Lys  Ser Val Gly Ser Arg  Trp Ala Val
    2510                2515                2520

Pro Glu  Asn Pro Cys Leu Val  Asn Glu Cys Val Arg  Val Glu Asp
    2525                2530                2535

Ala Val  Phe Val Gln Gln Arg  Asn Ile Ser Cys Pro  Gln Leu Ala
    2540                2545                2550

Val Pro  Thr Cys Pro Thr Gly  Phe Gln Leu Asn Cys  Glu Thr Ser
    2555                2560                2565

Glu Cys  Cys Pro Ser Cys His  Cys Glu Pro Val Glu  Ala Cys Leu
    2570                2575                2580

Leu Asn  Gly Thr Ile Ile Gly  Pro Gly Lys Ser Val  Met Val Asp
    2585                2590                2595

Leu Cys  Thr Thr Cys Arg Cys  Ile Val Gln Thr Asp  Ala Ile Ser
    2600                2605                2610

Arg Phe  Lys Leu Glu Cys Arg  Lys Thr Thr Cys Glu  Ala Cys Pro
    2615                2620                2625

Met Gly  Tyr Arg Glu Glu Lys  Ser Gln Gly Glu Cys  Cys Gly Arg
    2630                2635                2640

Cys Leu  Pro Thr Ala Cys Thr  Ile Gln Leu Arg Gly  Gly Arg Ile
    2645                2650                2655

Met Thr  Leu Lys Gln Asp Glu  Thr Phe Gln Asp Gly  Cys Asp Ser
    2660                2665                2670

His Leu  Cys Arg Val Asn Glu  Arg Gly Glu Tyr Ile  Trp Glu Lys
    2675                2680                2685

Arg Val  Thr Gly Cys Pro Pro  Phe Asp Glu His Lys  Cys Leu Ala
    2690                2695                2700

Glu Gly  Gly Lys Ile Val Lys  Ile Pro Gly Thr Cys  Cys Asp Thr
    2705                2710                2715

Cys Glu  Glu Pro Asp Cys Lys  Asp Ile Thr Ala Lys  Val Gln Tyr
    2720                2725                2730

Ile Lys  Val Gly Asp Cys Lys  Ser Gln Glu Glu Val  Asp Ile His
    2735                2740                2745

Tyr Cys  Gln Gly Lys Cys Ala  Ser Lys Ala Val Tyr  Ser Ile Asp
    2750                2755                2760

Ile Glu  Asp Val Gln Glu Gln  Cys Ser Cys Cys Leu  Pro Ser Arg
    2765                2770                2775

Thr Glu  Pro Met Arg Val Pro  Leu His Cys Thr Asn  Gly Ser Val
    2780                2785                2790

Val Tyr  His Glu Val Ile Asn  Ala Met Gln Cys Arg  Cys Ser Pro
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Arg Asn Cys Ser Lys
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<211> LENGTH: 8537
<212> TYPE: DNA
<213> ORGANISM: Mus musculus

<400> SEQUENCE: 9

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What is claimed is:

1. An isolated mutant human von Willebrand Factor A1 protein comprising one or more selected from the group consisting of: 1263 S>P, 1269D>N, 1274R>K, 1287R>M, 1302D>G, 1308R>H, 1313W>R, 1314V>I, 1326H>R, 1329I>L, 1330G>E, 1333D>A, 1344A>T, 1347V>I, 1350A>T, 1370S>G, 1379R>H, 1381A>T, 1385M>T 1391Q>P, 1394S>A, 1397F>L, 1421N>S, 1439V>L, 1442S>G, 1449Q>R, 1466P>A, 1469L>Q, 1472H>Q, 1473M>V, 1475Q>H, and 1479G>S, wherein each amino acid position corresponds to a position in SEQ ID NO: 6.

2. An isolated mutant human von Willebrand Factor A1 protein having SEQ ID NO: 6, wherein the protein comprises one or more mutation(s) selected from the group consisting of: 1263S>P, 1269D>N, 1274R>K, 1287R>M, 1302D>G, 1308R>H, 1313W>R, 1314V>I, 1326H>R, 1329I>L, 1330G>E, 1333D>A, 1344A>T, 1347V>I, 1350A>T, 1370S>G, 1379R>H, 1381A>T, 1385M>T, 1391Q>P, 1394S>A, 1397F>L, 1421N>S, 1439V>L, 1442S>G, 1449Q>R, 1466P>A, 1469L>Q, 1472H>Q, 1473M>V, 1475Q>H, and a 1479G>S.

3. An isolated mutant human von Willebrand Factor A1 protein comprising a 1326H>R mutation in an amino acid sequence of SEQ ID NO: 1.

4. A transgenic non-human animal expressing von Willebrand Factor A1 protein containing mutation(s) at one of more amino acid position selected from the group consisting of: 1263, 1269, 1274, 1287, 1302, 1308, 1313, 1314, 1326, 1329, 1330, 1333, 1344, 1347, 1350, 1370, 1379, 1381, 1385, 1391, 1394, 1397, 1421, 1439, 1442, 1449, 1466, 1469, 1472, 1473, 1475, and 1479, wherein the position corresponds to an amino acid position of human von Willebrand Factor A1 protein shown in SEQ ID NO: 6.

5. The transgenic non-human animal of claim **4**, wherein the animal is a murine, a porcine, a canine, a feline, a rabbit, or a primate.

6. The transgenic non-human animal of claim **4**, wherein the protein comprises a single mutation.

7. The transgenic non-human animal of claim **4**, wherein the protein comprises two or more mutations.

8. The transgenic non-human animal of claim **4**, wherein the protein comprises at least one mutation selected from the group consisting of: 1263>S, 1269>D, 1274>R, 1287>R, 1302>D, 1308>R, 1313R>W, 1314>V, 1326>H, 1329>I, 1330>G, 1333>D, 1344>A, 1347>V, 1350>A, 1370>S, 1379>R, 1381>A, 1385>M 1391>Q, 1394>S, 1397>F, 1421>N, 1439>V, 1442>S, 1449>Q, 1466>P, 1469>L, 1472>H, 1473>M, 1475>Q, 1479>G, and any combination thereof.

9. The transgenic non-human animal of claim **4**, wherein the protein comprises a 1326R>H mutation, a 1314I>V mutation, or a combination thereof.

10. The transgenic non-human animal of claim **8** or **9**, wherein the animal is a mouse.

11. The transgenic non-human animal of claim **8** or **9**, wherein the protein comprises SEQ ID NO: 5.

12. The transgenic non-human animal of claim **4**, wherein the VWF protein is at least 5%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 99%, or 100% identical to the A1 domain of human VWF protein as shown in SEQ ID NO: 1.

13. The transgenic non-human animal of claim **12**, wherein the von Willebrand Factor A1 protein of the transgenic animal contains the human A1 domain shown in SEQ ID NO: 1.

14. The transgenic non-human animal of claim **4**, wherein the von Willebrand Factor A1 protein is partially or completely replaced with a human von Willebrand Factor A1 protein comprising SEQ ID NO: 1.

15. The transgenic non-human animal of claim **4**, wherein the animal is a model for pre-clinical testing of compounds that expresses a mutant von Willebrand Factor (VWF) A1 protein containing one or more mutations, wherein the binding specificity of the mutant VWF-A1 protein changes from being specific for the animal platelets to being specific for human platelets.

16. The transgenic non-human animal of claim **15**, wherein the mutant VWF-A1 protein in the animal binds to human platelets.

17. A method for identifying a compound that modulates binding of VWF-A1 protein to GPIb-alpha protein, the method comprising:

a) providing an electronic library of test compounds;

b) providing atomic coordinates listed in Table 8 for at least 10 amino acid residues for the A1 domain of the VWF protein, wherein the coordinates have a root mean square deviation therefrom, with respect to at least 50% of Cα atoms, of not greater than about 2.5 Å, in a computer readable format;

c) converting the atomic coordinates into electrical signals readable by a computer processor to generate a three dimensional model of the VWF-A1 domain;

d) performing a data processing method, wherein electronic test compounds from the library are superimposed upon the three dimensional model of the VWF-A1 domain; and

e) determining which test compound fits into the binding pocket of the three dimensional model of the VWF-A1 protein,

thereby identifying which compound would modulate the binding of VWF-A1 protein to GPIb-alpha protein.

18. A method for identifying a compound that modulates binding of VWF-A1 protein to GPIb-alpha protein, the method comprising:

a) providing an electronic library of test compounds;

b) providing atomic coordinates listed in Table 8 in a computer readable format for at least 10, 15, 20, 25, 30, 35, or 40 amino acid residues for the A1 domain of the VWF protein, wherein the residues comprise two or more of the following residues: Pro1391, Arg1392, Arg1395, Val1398, Arg1399, Gln1402, Lys1406, Lys1423, Gln1424, Leu1427, Lys1430, or Glu1431;

c) converting the atomic coordinates into electrical signals readable by a computer processor to generate a three dimensional model of the VWF-A1 domain;

d) performing a data processing method, wherein electronic test compounds from the library are superimposed upon the three dimensional model of the VWF-A1 domain; and

e) determining which test compound fits into the binding pocket of the three dimensional model of the VWF-A1 protein,

thereby identifying which compound would modulate the binding of VWF-A1 protein to GPIb-alpha protein.

19. The method of claim **17** or **18**, wherein determining comprises detecting an IC_{50} of less than about 7.5 μg/ml for a test compound.

20. The method of claim **17** or **18**, further comprising:

f) obtaining or synthesizing a compound;

g) contacting VWF-A1 protein with the compound under a condition suitable for GPIb-alpha-VWF-A1 binding; and

h) determining whether the compound modulates GPIb-alpha-VWF-A1 protein binding using a diagnostic assay.

21. The method of claim **20**, wherein contacting comprises perfusing platelets into a flow chamber at a shear flow rate of at least 100 s^{-1}, wherein mutant murine VWF-A1 protein is immobilized on a bottom surface of the chamber.

22. The method of claim **20**, wherein contacting comprises perfusing platelets into the transgenic non-human animal of claim **4**.

23. The method of claim **21**, wherein contacting occurs sequentially.

24. The method of claim **21**, wherein the perfusing of platelets occurs prior to administration of the compound.

25. The method of claim **21**, wherein the platelets are human platelets.

26. The method of claim **21**, wherein the platelets are not murine platelets.

27. The method of claim **20**, wherein the determining comprises detecting an increase or decrease in the dissociation rate between VWF-A1 protein and GPIb-alpha protein by at least two-fold.

28. The method of claim **20**, wherein the determining comprises detecting an increase or decrease of platelet adhesion to a surface expressing VWF-A1 protein.

29. The method of claim **20**, wherein the determining comprises detecting an increase or decrease in a stabilization of an interaction between VWF-A1 protein and GPIb-alpha protein.

30. The method of claim **20**, wherein the determining comprises detecting thrombosis formation.

31. The method of claim **20**, wherein the determining comprises identifying an occurrence of an abnormal thrombotic event in the subject.

32. The method of claim **31**, wherein an abnormal thrombotic event comprises abnormal bleeding, abnormal clotting, death, or a combination thereof.

33. The method of claim **20**, wherein the determining comprises dynamic force microscopy, a coagulation factor assay, a platelet adhesion assay, thrombus imaging, a bleeding time assay, aggregometry, review of real-time video of blood flow, a Doppler ultrasound vessel occlusion assay, or a combination thereof.

34. The method of claim **21**, wherein perfusing platelets is followed by perfusion of a labeled agent.

35. The method of claim **34**, wherein the labeled agent comprises one or more of a nanoparticle, a fluorophore, a quantum dot, a microcrystal, a radiolabel, a dye, a gold biolabel, an antibody, or a small molecule ligand.

36. The method of claim **34**, wherein the agent targets a platelet receptor, a VWF protein, or a portion thereof.

37. A nucleic acid encoding the protein of any of claims **1-3**.

38. A vector encoding the nucleic acid of claim **37**.

39. An animal expressing the protein of any of claims **1-3**.

40. A method for treating von Willebrand Disease (VWD) in a subject in need thereof, the method comprising administering to the subject an effective amount of a compound that promotes platelet adhesion in the subject, wherein the compound increases the dissociation rate between VWF-A1 protein and GPIb-alpha protein by at least two-fold, thereby administration of the compound increases blood coagulation in the subject.

41. The method of claim **40**, wherein coagulation is measured by a coagulation factor assay, an ex-vivo flow chamber assay, or a combination thereof.

42. A method for rapidly detecting an internal vascular injury site in a subject, the method comprising:

a) administering to a subject a targeted molecular imaging agent, wherein the molecule circulates for an effective period of time in order to bind to the injury site within the subject;

b) tracking a deposition of the labeled targeted molecular imaging agent in the subject; and

c) identifying the site of a thrombus formation in the subject by imaging the targeted molecular imaging agent,

thereby the deposition of the targeted molecular imaging agent at the internal vascular injury site is indicative of internal bleeding within a subject.

43. The method of claim **42**, wherein the targeted molecular imaging agent is administered by subcutaneous, intramuscular, intra-peritoneal, or intravenous injection; infusion; by oral, nasal, or topical delivery; or a combination thereof.

44. The method of claim **42**, wherein the targeted molecular imaging agent comprises a nanoparticle, a fluorophore, a quantum dot, a microcrystal, a radiolabel, a dye, a gold biolabel, an antibody, a peptide, a small molecule ligand, or a combination thereof.

45. The method of claim **44**, wherein the nanoparticle comprises a perfluorocarbon.

46. The method of claim **44**, wherein the nanoparticle is coupled to an antibody, a small molecule, a peptide, or a receptor trap.

47. The method of claim **42**, wherein the targeted molecular imaging agent specifically binds to a platelet receptor, or a VWF protein, or a portion thereof.

48. The method of claim **42**, wherein the targeted molecular imaging agent has a $T_{1/2}$ of at least 30 minutes.

49. The method of claim **42**, wherein imaging comprises a PET scan, MRI, IR scan, ultrasound, nuclear imaging, or a combination thereof.

50. The method of claim **42**, wherein the subject is further administered a thrombotic compound.

51. The method of claim **50**, wherein the compound increases the dissociation rate between VWF-A1 protein and GPIb-alpha protein by at least two-fold.

52. A method for determining whether platelet function or morphology in a subject is abnormal, the method comprising:

a) affixing a molecule comprising a murine VWF-A1 domain to a surface of a flow chamber, wherein the domain comprises at least one mutation at a position selected from the group consisting of 1263>S, 1269>D, 1274>R, 1287>R, 1302>D, 1308>R, 1313R>W, 1314>V, 1326>H, 1329>I, 1330>G, 1333>D, 1344>A, 1347>V, 1350>A, 1370>S, 1379>R, 1381>A, 1385>M 1391>Q, 1394>S, 1397>F, 1421>N, 1439>V, 1442>S, 1449>Q, 1466>P, 1469>L, 1472>H, 1473>M, 1475>Q, 1479>G, and any combination thereof, where the position corresponds to an amino acid position of human von Willebrand Factor A1 protein shown in SEQ ID NO: 6;

b) perfusing through the flow chamber a volume of blood or plasma from a subject at a shear flow rate of at least about 100 s^{-1};

c) perfusing a targeted molecular imaging agent into the flow chamber; and

d) comparing the flow rate of the blood or plasma from the subject as compared to a normal flow rate, so as to determine whether the subject's platelet function or morphology is abnormal.

53. The method of claim **52**, wherein the affixing comprises (i) affixing an antibody which specifically binds VWF-A1 domain, and (ii) perfusing murine mutant VWF-A1 protein in the flow chamber at a shear flow rate of at least 100 s^{-1}.

54. The method of claim **52**, wherein the targeted molecular imaging agent comprises a nanoparticle, a fluorophore, a quantum dot, a microcrystal, a radiolabel, a dye, a gold biolabel, an antibody, a peptide, a small molecule ligand, or a combination thereof.

55. The method of claim **52**, wherein the targeted molecular imaging agent binds to a platelet receptor, a platelet ligand, or any region of a VWF protein or a portion thereof.

56. The method of claim **52**, wherein the targeted molecular imaging agent comprises horseradish peroxidase (HRP) coupled to an antibody directed at VWF-A1.

57. The method of claim **52**, wherein the comparing comprises a platelet adhesion assay, fluorescence imaging, a chromogenic indicator assay, a microscopy morphology analysis, or any combination thereof.

58. The method of claim **52**, wherein platelets bound to VWF-A1 are less than about 500 cells/mm^2.

59. The method of claim **58**, wherein the platelets are substantially spherical.

60. The method of claim **40**, **42**, or **52**, wherein the subject is a human, a canine, a feline, a murine, a porcine, an equine, or a bovine.

61. The method of claim **52**, wherein the VWF molecule is an antibody, a peptide, or a Fab fragment directed to a VWF polypeptide or a portion thereof.

62. A method for producing mutant von Willebrand Factor A1 protein that specifically binds human platelets, the method comprising:

(a) providing an animal expressing a mutant von Willebrand Factor A1 protein, wherein the mutation causes the platelet binding specificity of the animal von Willebrand Factor A1 protein to change to be specific for human platelets; and

(b) harvesting the mutant animal von Willebrand Factor A1 so as to produce von Willebrand Factor A1 protein that specifically binds human platelets.

63. The method of claim **62**, wherein the mutant animal von Willebrand Factor A1 protein comprises at least one mutation comprising 1263P>S, 1269N>D, 1274K>R, 1287M>R, 1302G>D, 1308H>R, 1313R>W, 1314I>V, 1326R>H, 1329L>1,1330E>G, 1333A>D, 1344T>A, 1347I>V, 1350T>A, 1370G>S, 1379H>R, 1381T>A, 1385T>M 1391P>Q, 1394A>S, 1397L>F, 1421S>N, 1439L>V, 1442G>S, 1449R>Q, 1466A>P, 1469Q>L, 1472Q>H, 1473V>M, 1475H>Q, 1479S>G, or any combination thereof.

64. A method for testing efficacy and toxicity of a gene therapy vector, the method comprising:

a) introducing a gene therapy vector into the animal of claim **4**, allowing sufficient time for expression of the vector;

b) perfusing platelets from a subject into the animal under a condition suitable for GPIb-alpha-VWF-A1 protein binding; and

c) determining whether or not a thrombotic event occurs in the animal.

65. The method of claim **64**, wherein the vector comprises a nucleic acid encoding a platelet receptor polypeptide, a platelet ligand polypeptide, or a VWF polypeptide, or a portion thereof.

66. The method of claim **64**, wherein the subject is a human, a dog, a cat, a horse, a pig, or a primate.

67. The method of claim **64**, wherein the platelets are not murine platelets.

68. The method of claim **64**, wherein the thrombotic event comprises blood clotting, abnormal bleeding, abnormal clotting, death, or a combination thereof.

69. The method of claim **64**, wherein the determining comprises dynamic force microscopy, a coagulation factor assay, a platelet adhesion assay, thrombus imaging, a bleeding time assay, aggregometry, review of real-time video of blood flow, a Doppler ultrasound vessel occlusion assay, or a combination thereof.

70. The method of claim **64**, wherein perfusing platelets is followed by perfusion of a labeled agent.

71. The method of claim **70**, wherein the labeled agent comprises one or more of a nanoparticle, a fluorophore, a quantum dot, a microcrystal, a radiolabel, a dye, a gold biolabel, an antibody, or a small molecule ligand.

72. The method of claim **71**, wherein the agent targets a platelet receptor, a VWF protein, or a portion thereof.

73. A method for calibrating an aggregometry device or a device for measuring clot formation or retraction, the method comprising:

a) providing hematologic data obtained from a subject, wherein blood or platelets from the subject is assessed by the device;

b) determining whether or not a thrombotic event occurs in the animal of claim **4**, wherein the animal is perfused with a sample of blood or platelets from the subject; and

c) correlating data obtained from (b) with the data obtained in (a) so as to calibrate the device, wherein a certain data obtained from the device is indicative of the corresponding thrombotic outcome determined in the animal of claim **4**.

74. The method of claim **73**, wherein the thrombotic event comprises blood clotting, abnormal bleeding, abnormal clotting, death, or a combination thereof.

75. The method of claim **22**, wherein contacting occurs sequentially.

76. The method of claim **22**, wherein the perfusing of platelets occurs prior to administration of the compound.

77. The method of claim **22**, wherein the platelets are human platelets.

78. The method of claim **22**, wherein the platelets are not murine platelets.

79. The method of claim **22**, wherein perfusing platelets is followed by perfusion of a labeled agent.

80. The method of claim **79**, wherein the labeled agent comprises one or more of a nanoparticle, a fluorophore, a quantum dot, a microcrystal, a radiolabel, a dye, a gold biolabel, an antibody, or a small molecule ligand.

81. The method of claim **79**, wherein the agent targets a platelet receptor, a VWF protein, or a portion thereof.

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