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DESCRIPTION

FIELD OF THE INVENTION

[0001] The present invention relates to polypeptides comprising modified truncated von Willebrand Factor which exhibits improved binding affinity to Factor VIII. The invention further relates to a complex comprising the polypeptide and FVIII, to polynucleotides encoding the polypeptides of the invention and methods of producing the polypeptides. Furthermore, the invention concerns the therapeutic or prophylactic use of the polypeptide or complex of the invention for treating blood coagulation disorders.

BACKGROUND OF THE INVENTION

[0002] There are various bleeding disorders caused by deficiencies of blood coagulation factors. The most common disorders are hemophilia A and B, resulting from deficiencies of blood coagulation factor VIII and IX, respectively. Another known bleeding disorder is von Willebrand's disease.

[0003] In plasma FVIII exists predominantly in a noncovalent complex with VWF and acts as a cofactor for activated factor IX in the membrane bound activated factor X generating complex.

[0004] Several attempts have been made to prolong the half-life of non-activated FVIII either by reducing its interaction with cellular receptors (WO 03/093313A2, WO 02/060951A2), by covalently attaching polymers to FVIII (WO 94/15625, WO 97/11957 and US 4970300), by encapsulation of FVIII (WO 99/55306), by introduction of novel metal binding sites (WO 97/03193), by covalently attaching the A2 domain to the A3 domain either by peptidic (WO 97/40145 and WO 03/087355) or disulfide linkage (WO 02/103024A2) or by covalently attaching the A1 domain to the A2 domain (WO2006/108590).

[0005] Another approach to enhance the functional half-life of FVIII or VWF is by PEGylation of FVIII (WO 2007/126808, WO 2006/053299, WO 2004/075923). PEGylation of VWF (WO 2006/071801) has also been attempted in an effort to indirectly enhance the half-life of FVIII present in plasma. Also fusion proteins of FVIII have been described (WO 2004/101740, WO2008/077616 and WO 2009/156137).

[0006] VWF, which is missing, functionally defective or only available in reduced quantity in different forms of von Willebrand disease (VWD), is a multimeric adhesive glycoprotein present in plasma, which has multiple physiological functions. During primary hemostasis VWF acts as a mediator between specific receptors on the platelet surface and components of the extracellular matrix such as collagen. Moreover, VWF serves as a carrier and stabilizing protein for procoagulant FVIII. VWF is synthesized in endothelial cells and megakaryocytes as

a 2813 amino acid precursor molecule. The amino acid sequence and the cDNA sequence of wild-type VWF are disclosed in Collins et al. 1987, Proc Natl. Acad. Sci. USA 84:4393-4397. The precursor polypeptide, pre-pro-VWF, consists of a 22-residue signal peptide, a 741-residue pro-peptide and the 2050-residue polypeptide found in plasma (Fischer et al., FEBS Lett. 351: 345-348, 1994). After cleavage of the signal peptide in the endoplasmic reticulum a C-terminal disulfide bridge is formed between two monomers of VWF. During further transport through the secretory pathway 12 N-linked and 10 O-linked carbohydrate side chains are added. Importantly, VWF dimers are multimerized via N-terminal disulfide bridges and the propeptide of 741 amino acids is cleaved off by the enzyme PACE/furin in the late Golgi apparatus. The propeptide as well as the high-molecular-weight multimers of VWF (VWF-HMWM) are stored in the Weibel-Pallade bodies of endothelial cells or in the α -Granules of platelets.

[0007] Once secreted into plasma the protease ADAMTS13 cleaves VWF within the A1 domain of VWF. Plasma VWF consists of a range of multimers ranging from single dimers of 500 kDa to multimers consisting of more than 20 dimers of a molecular weight of over 10,000 kDa. Typically VWF high molecular weight multimers (VWF-HMWM) have the strongest hemostatic activity, which can be measured in ristocetin cofactor activity (VWF:RCo). The higher the ratio of VWF:RCo/VWF antigen, the higher the relative amount of high molecular weight multimers.

[0008] Defects in VWF are causal to von Willebrand disease (VWD), which is characterized by a more or less pronounced bleeding phenotype. VWD type 3 is the most severe form in which VWF is completely missing, VWD type 1 relates to a quantitative loss of VWF and its phenotype can be very mild. VWD type 2 relates to qualitative defects of VWF and can be as severe as VWD type 3. VWD type 2 has many sub forms some of them being associated with the loss or the decrease of high molecular weight multimers. Von VWD type 2a is characterized by a loss of both intermediate and large multimers. VWD type 2B is characterized by a loss of highest-molecular-weight multimers.

[0009] VWD is the most frequent inherited bleeding disorder in humans and can be treated by replacement therapy with concentrates containing VWF of plasma or recombinant origin. VWF can be prepared from human plasma as for example described in EP 05503991. EP 0784632 describes a method for producing and isolating recombinant VWF.

[0010] In plasma FVIII binds with high affinity to VWF, which protects it from premature catabolism and thus, plays in addition to its role in primary hemostasis, a crucial role in regulation of plasma levels of FVIII and as a consequence is also a central factor in the control of secondary hemostasis. The half-life of non-activated FVIII bound to VWF is about 12 to 14 hours in plasma. In von Willebrand disease type 3, where no or almost no VWF is present, the half-life of FVIII is only about 6 hours, leading to symptoms of mild to moderate hemophilia A in such patients due to decreased concentrations of FVIII. The stabilizing effect of VWF on FVIII has also been used to aid recombinant expression of FVIII in CHO cells (Kaufman et al. 1989, Mol Cell Biol).

[0011] The disclosure of Yee et al., 2014, Blood. Vol. 124 (3) p. 445-451 ("A von Willebrand factor fragment containing the D'D3 domains is sufficient to stabilize coagulation factor VIII in mice") relates to the finding that a truncated von Willebrand factor (VWF) fragment containing the factor VIII (FVIII)-binding D'D3 region of VWF is sufficient to stabilize endogenous FVIII levels in VWF-deficient mice. The VWF fragments were either expressed or infused into VWF deficient mice. However, coinfusion of a VWF D'D3-Fc with FVIII resulted in the rapid clearance of the exogenously administered FVIII in FVIII-deficient mice. The D'D3 constructs described in Yee et al. (2014) are monomers, dimers, or multimers. The D'D3 constructs shown in Yee et al. (2014) were not able to stabilize a co-administered FVIII in hemophilia A mice.

[0012] In WO2013/120939 A1 certain amino acid substitutions within the D' domain of a full length VWF have been suggested in order to increase the binding affinity to FVIII. The D' domain can have one, two, three, four, five or more mutations. According to this disclosure, the FVIII binding affinity of the VWF variants is increased by at least 10%.

[0013] The disclosure of Castro-Nunez et al., 2013, The Journal of Biological Chemistry. Vol. 288. No. 1, pp. 393-400, relates to the role of the N-terminus of VWF in FVIII binding. It was shown that a S764A substitution results in increased and a K773A substitution results in decreased FVIII binding, respectively. For both amino acid positions in VWF, opposite roles in the interaction with FVIII are proposed.

SUMMARY OF THE INVENTION

[0014] In the current applicant's co-pending International Patent Application no. PCT/AU2015/050369 it is disclosed that a number of modifications in domain D' of VWF can increase binding to Factor VIII. The present inventors have now found that the binding of VWF to Factor VIII can be increased by other modifications in D' and in particular by modifications in the D3 domain.

[0015] The present invention therefore relates to a polypeptide according to claim 1, a complex according to claim 12, a polypeptide for use according to claim 13 or claim 17, a pharmaceutical composition as defined in claim 14 or in claim 15, a pharmaceutical kit according to claim 16, a polynucleotide according to claim 18, a plasmid according to claim 19, a host cell according to claim 20, and a method according to claim 21. Further preferred embodiments are described in the dependent claims.

BRIEF DESCRIPTION OF FIGURES

[0016]

Figure 1: Sample sensorgrams from the screen at neutral pH. The two candidates with strongest affinity and slowest off rate are circled.

Figure 2: Sample sensorgrams showing detailed kinetics of CSL627 for two mutant D'D3-HSA candidates at pH7. a) Factor VIII binding D'D3-HSA with mutations: V1083A, S764G, S766Y; b) Factor VIII binding D'D3-HSA with mutations: S764G, S766Y; c) Factor VIII binding D'D3-HSA with mutations: V1083A, S764P, S766W; d) Factor VIII binding D'D3-HSA S764P, S766W.

Figure 3: Sample sensorgrams showing detailed kinetics of Factor VIII for two mutant D'D3-HSA candidates at pH5.5 a) Factor VIII binding D'D3-HSA with mutations: V1083A, S764G, S766Y; b) Factor VIII binding D'D3-HSA with mutations: S764G, S766Y. c) Factor VIII binding D'D3-HSA with mutations: V1083A, S764P, S766W d) Factor VIII binding D'D3-HSA S764P, S766W.

Figure 4: a) CSL627 binding D'D3-HSA Dimer with mutations: V1083A, S764E, S766Y at neutral pH ; b) CSL627 binding wildtype D'D3-HSA Dimer at neutral pH.

Figure 5: a) CSL627 binding D'D3-HSA Dimer with mutations: V1083A, S764E, S766Y b) CSL627 binding wildtype D'D3-HSA Dimer at pH5.5.

DETAILED DESCRIPTION

[0017] Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

[0018] It must be noted that, as used in the subject specification, the singular forms "a", "an" and "the" include plural aspects unless the context clearly dictates otherwise. Thus, for example, reference to "an agent" includes a single agent, as well as two or more agents; reference to "a molecule" includes a single molecule, as well as two or more molecules; and so forth.

Truncated VWF

[0019] The term "von Willebrand Factor" or "VWF", as used herein, refers to any polypeptide having a biological activity of wild type VWF, in particular the ability to bind Factor VIII. The gene encoding wild type VWF is transcribed into a 9 kb mRNA which is translated into a pre-propolypeptide of 2813 amino acids with an estimated molecular weight of 310,000 Da. The pre-propolypeptide contains a 22 amino acids signal peptide, a 741 amino acid pro-polypeptide and the mature subunit. Cleavage of the 741 amino acids propolypeptide from the N-terminus results in mature VWF consisting of 2050 amino acids. The amino acid sequence of the VWF pre-propolypeptide is shown in SEQ ID NO:2. Unless indicated otherwise, the amino acid

numbering of VWF residues in this application refers to SEQ ID NO:2, even if the VWF molecule does not need to comprise all residues of SEQ ID NO:2. The amino acid sequence of mature VWF is shown in SEQ ID NO:4. The term "VWF" as used herein refers to the mature form of VWF unless indicated otherwise.

[0020] The propolypeptide of wild type VWF comprises multiple domains which are arranged in the following order:

D1-D2-D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-CK

[0021] The D1 and D2 domain represent the propeptide which is cleaved off to yield the mature VWF. The D'-D3 domains encompass amino acids responsible for binding to Factor VIII. The amino acid sequence of at least a portion of D'-D3 domains of wild type VWF is shown in SEQ ID NO:3. The carboxy terminal 90 residues comprise the "CK" domain that is homologous to the "cysteine knot" superfamily of protein. These family members have a tendency to dimerise through disulfide bonds.

[0022] Preferably, wild type VWF comprises the amino acid sequence of mature VWF as shown in SEQ ID NO:4. Also encompassed are additions, insertions, N-terminal, C-terminal or internal deletions of VWF as long as a biological activity of VWF, in particular the ability to bind FVIII, is retained. The biological activity is retained in the sense of the invention if the VWF with deletions retains at least 10%, preferably at least 25%, more preferably at least 50%, most preferably at least 75% of the biological activity of wild-type VWF. The biological activity of wild-type VWF can be determined by the artisan using methods for ristocetin co-factor activity (Federici AB et al. 2004. Haematologica 89:77-85), binding of VWF to GP Ib α of the platelet glycoprotein complex Ib-V-IX (Sucker et al. 2006. Clin Appl Thromb Hemost. 12:305-310), or a collagen binding assay (Kallas & Talpsep. 2001. Annals of Hematology 80:466-471). Where the biological activity of VWF is the ability to bind FVIII this can be measured in a number of ways, however, it is preferably measured as described in Example 1 herein.

Factor VIII

[0023] The terms "blood coagulation Factor VIII", "Factor VIII" and "FVIII" are used interchangeably herein. "Blood coagulation Factor VIII" includes wild-type blood coagulation FVIII as well as derivatives of wild-type blood coagulation FVIII having the procoagulant activity of wild-type blood coagulation FVIII. Derivatives may have deletions, insertions and/or additions compared with the amino acid sequence of wild-type FVIII. The term FVIII includes proteolytically processed forms of FVIII, e.g. the form before activation, comprising heavy chain and light chain. Included are plasma derived and recombinant FVIII including B domain deleted FVIII. Examples of Commercial products include Advate[®], Kogenate[®], Xyntha[®], Loctate[®] and

Novoeight®.

[0024] The term "FVIII" includes any FVIII variants or mutants having at least 25%, more preferably at least 50%, most preferably at least 75% of the biological activity of wild-type factor VIII.

[0025] As non-limiting examples, FVIII molecules include FVIII mutants preventing or reducing APC cleavage (Amano 1998. *Thromb. Haemost.* 79:557-563), FVIII mutants further stabilizing the A2 domain (WO 97/40145), FVIII mutants having increased expression (Swaroop et al. 1997. *JBC* 272:24121-24124), FVIII mutants having reduced immunogenicity (Lollar 1999. *Thromb. Haemost.* 82:505-508), FVIII reconstituted from differently expressed heavy and light chains (Oh et al. 1999. *Exp. Mol. Med.* 31:95-100), FVIII mutants having reduced binding to receptors leading to catabolism of FVIII like HSPG (heparan sulfate proteoglycans) and/or LRP (low density lipoprotein receptor related protein) (Ananyeva et al. 2001. *TCM*, 11:251-257), disulfide bond-stabilized FVIII variants (Gale et al., 2006. *J. Thromb. Hemost.* 4:1315-1322), FVIII mutants with improved secretion properties (Miao et al., 2004. *Blood* 103:3412-3419), FVIII mutants with increased cofactor specific activity (Wakabayashi et al., 2005. *Biochemistry* 44:10298-304), FVIII mutants with improved biosynthesis and secretion, reduced ER chaperone interaction, improved ER-Golgi transport, increased activation or resistance to inactivation and improved half-life (summarized by Pipe 2004. *Sem. Thromb. Hemost.* 30:227-237). Another particularly preferred example is a recombinant form of FVIII as described in Zollner et al 2013, *Thrombosis Research*, 132:280-287.

[0026] Preferably FVIII comprises the full length sequence of FVIII as shown in SEQ ID NO:14. Also encompassed are additions, insertions, substitutions, N-terminal, C-terminal or internal deletions of FVIII as long as the biological activity of FVIII is retained. The biological activity is retained in the sense of the invention if the FVIII with modifications retains at least 10%, preferably at least 25%, more preferably at least 50%, most preferably at least 75% of the biological activity of wild-type FVIII. The biological activity of FVIII can be determined by the artisan as described below.

[0027] A suitable test to determine the biological activity of FVIII is for example the one stage or the two stage coagulation assay (Rizza et al. 1982. *Coagulation assay of FVIII:C and FIXa* in Bloom ed. *The Hemophilias*. NY Churchill Livingstone 1992) or the chromogenic substrate FVIII:C assay (S. Rosen, 1984. *Scand J Haematol* 33: 139-145, suppl.).

[0028] The amino acid sequence of the mature wild-type form of human blood coagulation FVIII is shown in SEQ ID NO:14. The reference to an amino acid position of a specific sequence means the position of said amino acid in the FVIII wild-type protein and does not exclude the presence of mutations, e.g. deletions, insertions and/or substitutions at other positions in the sequence referred to. For example, a mutation in "Glu2004" referring to SEQ ID NO:14 does not exclude that in the modified homologue one or more amino acids at positions 1 through 2332 of SEQ ID NO:14 are missing.

[0029] "FVIII" and/or "VWF" within the above definition also include natural allelic variations that may exist and occur from one individual to another. "FVIII" and/or "VWF" within the above definition further includes variants of FVIII and/or VWF. Such variants differ in one or more amino acid residues from the wild-type sequence. Examples of such differences may include conservative amino acid substitutions, i.e. substitutions within groups of amino acids with similar characteristics, e.g. (1) small amino acids, (2) acidic amino acids, (3) polar amino acids, (4) basic amino acids, (5) hydrophobic amino acids, and (6) aromatic amino acids. Examples of such conservative substitutions are shown in Table 1.

Table 1

(1)	Alanine	Glycine		
(2)	Aspartic acid	Glutamic acid		
(3)	Asparagine	Glutamine	Serine	Threonine
(4)	Arginine	Histidine	Lysine	
(5)	Isoleucine	Leucine	Methionine	Valine
(6)	Phenylalanine	Tyrosine	Tryptophan	

[0030] The feature "truncated" means that the polypeptide does not comprise the entire amino acid sequence of mature VWF (amino acids 764-2813 of SEQ ID NO:2). Typically, the truncated VWF does not comprise all amino acids 764-2813 of SEQ ID NO:2 but only a fragment thereof. A truncated VWF may also be referred to as a VWF fragment, or in the plural as VWF fragments.

[0031] Typically, the truncated VWF is capable of binding to a Factor VIII. Preferably, the truncated VWF is capable of binding to the mature form of human native Factor VIII. In another embodiment, the truncated VWF is capable of binding to the single-chain Factor VIII consisting of the amino acid sequence SEQ ID NO:15.

[0032] The truncated VWF of the present invention preferably comprises or consists of (a) an amino acid sequence having a sequence identity of at least 90% to amino acids 764 to 1242 of SEQ ID NO:2, or (b) a fragment thereof, provided that the truncated VWF is still capable of binding to FVIII. More preferably, the truncated VWF consists of (a) an amino acid sequence having a sequence identity of at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, to amino acids 764 to 1242 of SEQ ID NO:2, or (b) a fragment thereof, provided that the truncated VWF is still capable of binding to FVIII. Most preferably, the truncated VWF consists of (a) amino acids 764 to 1242 of SEQ ID NO:2, or (b) a fragment thereof, provided that the truncated VWF is still capable of binding to FVIII.

[0033] As described in more detail below, the polypeptide may be prepared by a method which uses cells comprising a nucleic acid encoding the polypeptide comprising the truncated VWF. The nucleic acid is introduced into suitable host cells by techniques that are well known to those skilled in the art.

[0034] In a preferred embodiment, the nucleic acid in the host cell encodes (a) an amino acid sequence having a sequence identity of at least 90% to amino acids 1 to 1242 of SEQ ID NO:2, or (b) a fragment thereof, provided that the truncated mature VWF is still capable of binding to FVIII. More preferably, the nucleic acid encodes (a) an amino acid sequence having a sequence identity of at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, to amino acids 1 to 1242 of SEQ ID NO:2, or (b) a fragment thereof, provided that the truncated VWF is still capable of binding to FVIII. Most preferably, the nucleic acid encodes (a) amino acids 1 to 1242 of SEQ ID NO:2, or (b) a fragment thereof, provided that the truncated VWF is still capable of binding to FVIII. Especially if the polypeptide in accordance with this invention is a dimer, the nucleic acid will comprise a sequence encoding amino acids 1 to 763 of VWF (e.g. SEQ ID NO:2), even if the truncated VWF in the polypeptide does not comprise amino acids 1 to 763 of VWF (e.g. SEQ ID NO:2).

[0035] In other embodiments the truncated VWF comprises or consists of one of the following amino acid sequences, each referring to SEQ ID NO:2: 776-805; 766-805; 764-805; 776-810; 766-810; 764-810; 776-815; 766-815; 764-815; 776-820; 766-820; 764-820; 776-825; 766-825; 764-825; 776-830; 766-830; 764-830; 776-835; 766-835; 764-835; 776-840; 766-840; 764-840; 776-845; 766-845; 764-845; 76-850; 766-850; 764-850; 776-855; 766-855; 764-855; 776-860; 766-860; 764-860; 776-864; 766-864; 764-864; 776-865; 766-865; 764-865; 776-870; 766-870; 764-870; 776-875; 766-875; 764-875; 776-880; 766-880; 764-880; 776-885; 766-885; 764-885; 776-890; 766-890; 764-890; 776-895; 766-895; 764-895; 776-900; 766-900; 764-900; 776-905; 766-905; 764-905; 776-910; 766-910; 764-910; 776-915; 766-915; 764-915; 776-920; 766-920; 764-920; 776-925; 766-925; 764-925; 776-930; 766-930; 764-930; 776-935; 766-935; 764-935; 776-940; 766-940; 764-940; 776-945; 766-945; 764-945; 776-950; 766-950; 764-950; 776-955; 766-955; 764-955; 776-960; 766-960; 764-960; 776-965; 766-965; 764-965; 776-970; 766-970; 764-970; 776-975; 766-975; 764-975; 776-980; 766-980; 764-980; 776-985; 766-985; 764-985; 776-990; 766-990; 764-990; 776-995; 766-995; 764-995; 776-1000; 766-1000; 764-1000; 776-1005; 766-1005; 764-1005; 776-1010; 766-1010; 764-1010; 776-1015; 766-1015; 764-1015; 776-1020; 766-1020; 764-1020; 776-1025; 766-1025; 764-1025; 776-1030; 766-1030; 764-1030; 776-1035; 766-1035; 764-1035; 776-1040; 766-1040; 764-1040; 776-1045; 766-1045; 764-1045; 776-1050; 766-1050; 764-1050; 776-1055; 766-1055; 764-1055; 776-1060; 766-1060; 764-1060; 776-1065; 766-1065; 764-1065; 776-1070; 766-1070; 764-1070; 776-1075; 766-1075; 764-1075; 776-1080; 766-1080; 764-1080; 776-1085; 766-1085; 764-1085; 776-1090; 766-1090; 764-1090; 776-1095; 766-1095; 764-1095; 776-1100; 766-1100; 764-1100; 776-1105; 766-1105; 764-1105; 776-1110; 766-1110; 764-1110; 776-1115; 766-1115; 764-1115; 776-1120; 766-1120; 764-1120; 776-1125; 766-1125; 764-1125; 776-1130; 766-1130; 764-1130; 776-1135; 766-1135; 764-1135; 776-1140; 766-1140; 764-1140; 776-1145; 766-1145; 764-1145; 776-1150; 766-1150; 764-1150; 776-1155; 766-1155; 764-1155; 776-1160; 766-1160; 764-1160; 776-1165; 766-1165; 764-1165; 776-1170; 766-1170; 764-1170; 776-1175; 766-1175; 764-1175; 776-1180; 766-1180; 764-1180; 776-1185; 766-1185; 764-1185; 776-1190; 766-1190; 764-1190; 776-1195; 766-1195; 764-1195; 776-1200; 766-1200; 764-1200; 776-1205; 766-1205; 764-1205; 776-1210; 766-1210; 764-1210; 776-1215; 766-1215; 764-1215; 776-1220; 766-1220; 764-1220; 776-1225; 766-1225; 764-1225; 776-1230; 766-1230; 764-1230; 776-1235; 766-1235;

764-1235; 776-1240; 766-1240; 764-1240; 776-1242; 766-1242; 764-1242; 764-1247; 764-1464; 764-1250; 764-1041; 764-828; 764-865; 764-1045; 764-1035; 764-1128; 764-1198; 764-1268; 764-1270; 764-1261; 764-1264; 764-1459; 764-1463; 764-1464; 764-1683; 764-1873; 764-1482; 764-1479; 764-1672; and 764-1874.

[0036] In certain embodiments the truncated VWF has an internal deletion relative to mature wild type VWF. For example, the A1, A2, A3, D4, C1, C2, C3, C4, C5, C6 domains or combinations thereof may be deleted, and the D' domain, the D3 domain and the CK domain is retained. In further embodiments the truncated VWF does not comprise the binding sites for platelet glycoprotein Iba (GPIba), collagen and/or integrin $\alpha\text{IIb}\beta\text{III}$ (RGDS sequence within the C1 domain). In other embodiments, the truncated VWF does not comprise the cleavage site (Tyr1605-Met1606) for ADAMTS13 which is located at the central A2 domain of VWF. In yet another embodiment, the truncated VWF does not comprise the binding sites for GPIba, and/or does not comprise the binding site for collagen, and/or does not comprise the binding site for integrin $\alpha\text{IIb}\beta\text{III}$, and/or it does not comprise the cleavage site (Tyr1605-Met1606) for ADAMTS13 which is located at the central A2 domain of VWF.

[0037] In other embodiments the truncated VWF comprises or consists of an amino acid sequence that has a sequence identity of at least 90%, or at least 91%, or at least 92%, or at least 93%, or at least 94%, or at least 95%, or at least 96%, or at least 97%, or at least 98%, or at least 99%, to one of the amino acid sequences recited in the preceding paragraph, provided that the truncated VWF is capable of binding to FVIII.

[0038] A polypeptide of the invention is termed a "dimer" in the present invention if two monomers of polypeptide of the invention are linked covalently. Preferably the two monomeric subunits are covalently linked via at least one disulfide bridge, e.g. by one, two, three or four disulfide bridges. The cysteine residues forming the at least one disulfide bridge are preferably located within the truncated VWF portion of the polypeptide of the invention. In one embodiment, these cysteine residues are Cys-1099, Cys-1142, Cys-1222, Cys-1225, or Cys-1227 or combinations thereof.

[0039] If the polypeptide of the invention is a dimer, the truncated VWF preferably comprises or consists of two polypeptides each with an amino acid sequence having a sequence identity of at least 90% to amino acids 764 to 1099, amino acids 764 to 1142, amino acids 764 to 1222, amino acids 764 to 1225, amino acids 764 to 1227, amino acids 764 to 1242, amino acids 764 to 1247, or amino acids 764 to 1270 of SEQ ID NO:2 and is capable of binding to FVIII. In preferred embodiments the truncated VWF comprises or consists of an amino acid sequence having a sequence identity of at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, to amino acids 764 to 1099, amino acids 764 to 1142, amino acids 764 to 1222, amino acids 764 to 1225, or amino acids 764 to 1227 of SEQ ID NO:2 and is capable of binding to FVIII. Most preferably, the truncated VWF comprises or consists of amino acids 764 to 1099, amino acids 764 to 1142, amino acids 764 to 1222, amino acids 764 to 1225, amino acids 764 to 1227, amino acids 764 to 1242, amino acids 764 to 1247, or amino acids 764 to 1270 of SEQ ID NO:2.

[0040] The truncated VWF may be any one of the VWF fragments disclosed in WO 2013/106787, WO 2014/198699, WO 2011/060242, WO 2014/011819, WO 2013/083858, WO 2015/185758 or WO 2013/093760.

Half-life extending moiety

[0041] In addition to the truncated VWF, the polypeptide of the invention may further comprise a half-life extending moiety. The half-life-extending moiety may be a heterologous amino acid sequence fused to the truncated VWF. Alternatively, the half-life-extending moiety may be chemically conjugated to the polypeptide comprising the truncated VWF by a covalent bond different from a peptide bond.

[0042] In certain embodiments of the invention, the half-life of the polypeptide of the invention is extended by chemical modification, e.g. attachment of a half-life extending moiety such as polyethylene glycol (PEGylation), glycosylated PEG, hydroxyl ethyl starch (HESylation), polysialic acids, elastin-like polypeptides, heparosan polymers or hyaluronic acid. In another embodiment, the polypeptide of the invention is conjugated to a HLEP such as albumin via a chemical linker. The principle of this conjugation technology has been described in an exemplary manner by Conjuchem LLC (see, e.g., US patent No. 7,256,253).

Half-life enhancing polypeptides (HLEPs)

[0043] Preferably, the half-life extending moiety is a half-life extending polypeptide (HLEP), more preferably HLEP is selected from albumin or fragments thereof, immunoglobulin constant region and portions thereof, e.g. the Fc fragment, solvated random chains with large hydrodynamic volume (e.g. XTEN (Schellenberger et al. 2009; Nature Biotechnol. 27:1186-1190), homo-amino acid repeats (HAP) or proline-alanine-serine repeats (PAS), afamin, alpha-fetoprotein, Vitamin D binding protein, transferrin or variants thereof, carboxyl-terminal peptide (CTP) of human chorionic gonadotropin- β subunit, polypeptides or lipids capable of binding under physiological conditions to albumin or immunoglobulin constant region.

[0044] A "half-life enhancing polypeptide" as used herein is preferably selected from the group consisting of albumin, a member of the albumin-family, the constant region of immunoglobulin G and fragments thereof, region and polypeptides capable of binding under physiological conditions to albumin, to members of the albumin family as well as to portions of an immunoglobulin constant region. It may be a full-length half-life-enhancing protein described herein (e.g. albumin, a member of the albumin-family or the constant region of immunoglobulin G) or one or more fragments thereof that are capable of stabilizing or prolonging the therapeutic activity or the biological activity of the coagulation factor. Such fragments may be of 10 or more amino acids in length or may include at least about 15, at least about 20, at least about 25, at least about 30, at least about 50, at least about 100, or more contiguous amino

acids from the HLEP sequence or may include part or all of specific domains of the respective HLEP, as long as the HLEP fragment provides a functional half-life extension of at least 25% compared to the respective polypeptide without the HLEP.

[0045] The HLEP portion of the polypeptide of the invention may be a variant of a wild type HLEP. The term "variants" includes insertions, deletions and substitutions, either conservative or non-conservative, where such changes do not substantially alter the FVIII-binding activity of the truncated VWF.

[0046] In particular, the proposed VWF HLEP fusion constructs of the invention may include naturally occurring polymorphic variants of HLEPs and fragments of HLEPs. The HLEP may be derived from any vertebrate, especially any mammal, for example human, monkey, cow, sheep, or pig. Non-mammalian HLEPs include, but are not limited to, hen and salmon.

[0047] In one embodiment the polypeptide has the following structure:

tVWF - L1 - H, [formula 1]

wherein tVWF is the truncated VWF, L1 is a chemical bond or a linker sequence, and H is a HLEP.

[0048] L1 may be a chemical bond or a linker sequence consisting of one or more amino acids, e.g. of 1 to 50, 1 to 30, 1 to 20, 1 to 15, 1 to 10, 1 to 5 or 1 to 3 (e.g. 1, 2 or 3) amino acids and which may be equal or different from each other. Usually, the linker sequences are not present at the corresponding position in the wild-type VWF. Examples of suitable amino acids present in L1 include Gly and Ser. The linker should be non-immunogenic and may be a non-cleavable or cleavable linker. Non-cleavable linkers may be comprised of alternating glycine and serine residues as exemplified in WO2007/090584. In another embodiment of the invention the peptidic linker between the truncated VWF moiety and the albumin moiety consists of peptide sequences, which serve as natural interdomain linkers in human proteins. Preferably such peptide sequences in their natural environment are located close to the protein surface and are accessible to the immune system so that one can assume a natural tolerance against this sequence. Examples are given in WO2007/090584. Cleavable linker sequences are described, e.g., in WO 2013/120939 A1.

[0049] Preferred HLEP sequences are described infra. Likewise encompassed by the invention are fusions to the exact "N-terminal amino acid" or to the exact "C-terminal amino acid" of the respective HLEP, or fusions to the "N-terminal part" or "C-terminal part" of the respective HLEP, which includes N-terminal deletions of one or more amino acids of the HLEP. The polypeptide may comprise more than one HLEP sequence, e.g. two or three HLEP sequences. These multiple HLEP sequences may be fused to the C-terminal part of VWF in tandem, e.g. as successive repeats.

Albumin as HLEP

[0050] The terms, "human serum albumin" (HSA) and "human albumin" (HA) and "albumin" (ALB) are used interchangeably in this application. The terms "albumin" and "serum albumin" are broader, and encompass human serum albumin (and fragments and variants thereof) as well as albumin from other species (and fragments and variants thereof).

[0051] As used herein, "albumin" refers collectively to albumin polypeptide or amino acid sequence, or an albumin fragment or variant, having one or more functional activities (e.g., biological activities) of albumin. In particular, "albumin" refers to human albumin or fragments thereof, especially the mature form of human albumin as shown in SEQ ID NO:16 herein or albumin from other vertebrates or fragments thereof, or analogs or variants of these molecules or fragments thereof.

[0052] In particular, the proposed polypeptides of the invention may include naturally and non-naturally occurring polymorphic variants of human albumin and fragments of human albumin. Generally speaking, an albumin fragment or variant will be at least 10, preferably at least 40, most preferably more than 70 amino acids long.

[0053] Preferred embodiments of the invention include albumin variants used as a HLEP of the polypeptide of the invention with enhanced binding to the FcRn receptor. Such albumin variants may lead to a longer plasma half-life of a truncated VWF albumin variant fusion protein as compared to a truncated VWF fusion with a wild-type albumin. Variants include those described in WO 2014072481, WO 2012150319, WO 2013135896, WO 2011124718, WO 2011051489 and WO 2012059486.

[0054] The albumin portion of the polypeptides of the invention may comprise at least one subdomain or domain of HA or conservative modifications thereof.

Immunoglobulins as HLEPs

[0055] Immunoglobulin G (IgG) constant regions (Fc) are known in the art to increase the half-life of therapeutic proteins (Dumont J A et al. 2006. BioDrugs 20:151-160). The IgG constant region of the heavy chain consists of 3 domains (CH1-CH3) and a hinge region. The immunoglobulin sequence may be derived from any mammal, or from subclasses IgG1, IgG2, IgG3 or IgG4, respectively. IgG and IgG fragments without an antigen-binding domain may also be used as HLEPs. The therapeutic polypeptide portion is connected to the IgG or the IgG fragments preferably via the hinge region of the antibody or a peptidic linker, which may even be cleavable. Several patents and patent applications describe the fusion of therapeutic proteins to immunoglobulin constant regions to enhance the therapeutic protein's in vivo half-lives. US 2004/0087778 and WO 2005/001025 describe fusion proteins of Fc domains or at least portions of immunoglobulin constant regions with biologically active peptides that increase the half-life of the peptide, which otherwise would be quickly eliminated in vivo. Fc-IFN- β fusion proteins were described that achieved enhanced biological activity, prolonged circulating half-life and greater solubility (WO 2006/000448). Fc-EPO proteins with a prolonged serum half-life

and increased in vivo potency were disclosed (WO 2005/063808) as well as Fc fusions with G-CSF (WO 2003/076567), glucagon-like peptide-1 (WO 2005/000892), clotting factors (WO 2004/101740) and interleukin-10 (U.S. Pat. No. 6,403,077), all with half-life enhancing properties.

[0056] Various HLEPs which can be used in accordance with this invention are described in detail in WO 2013/120939 A1.

Linker sequences

[0057] According to this invention, the therapeutic polypeptide moiety may be coupled to the HLEP moiety by a peptide linker. The linker should be non-immunogenic and may be a non-cleavable or cleavable linker.

[0058] Non-cleavable linkers may be comprised of alternating glycine and serine residues as exemplified in WO2007/090584.

[0059] In another embodiment of the invention the peptidic linker between the VWF moiety and the albumin moiety consists of peptide sequences, which serve as natural interdomain linkers in human proteins. Preferably such peptide sequences in their natural environment are located close to the protein surface and are accessible to the immune system so that one can assume a natural tolerance against this sequence. Examples are given in WO2007/090584.

[0060] Cleavable linkers should be flexible enough to allow cleavage by proteases. In a preferred embodiment the cleavage of the linker proceeds comparably fast as the activation of FVIII within the fusion protein, if the fusion protein is a modified FVIII.

[0061] The cleavable linker preferably comprises a sequence derived from

1. (a) the therapeutic polypeptide to be administered itself if it contains proteolytic cleavage sites that are proteolytically cleaved during activation of the therapeutic polypeptide,
2. (b) a substrate polypeptide cleaved by a protease which is activated or formed by the involvement of the therapeutic polypeptide, or
3. (c) a polypeptide involved in coagulation or fibrinolysis.

[0062] The linker region in a more preferred embodiment comprises a sequence of VWF, which should result in a decreased risk of neoantigenic properties of the expressed fusion protein.

[0063] The linker peptides are preferably cleavable by the proteases of the coagulation system, for example FIIa, FIXa, FXa, FXIa, FXIIa and FVIIa.

[0064] Exemplary combinations of therapeutic polypeptide, cleavable linker and HLEP include the constructs listed in WO2007/090584 (for example in table 2 and figure 4) and WO2007/144173 (for example in table 3a and 3b), but are not limited to these.

[0065] In another embodiment, the functional half-life of polypeptide of the invention or of FVIII complexed with the polypeptide of the invention is prolonged compared to that of wild type VWF or to that of FVIII complexed with wild type VWF, or with the reference polypeptide as defined supra. The increase may be more than 15%, for example at least 20% or at least 50%. Again, such functional half-life values can be measured in vitro in blood samples taken at different time intervals from said mammal after the modified VWF or the complex of FVIII with modified VWF has been administered.

[0066] In another embodiment of the invention, the polypeptide of the invention or FVIII complexed with the polypeptide of the invention exhibits an improved in vivo recovery compared to wild type VWF or to FVIII complexed with wild type VWF, or with the reference polypeptide defined supra. The in vivo recovery can be determined in vivo for example in normal animals or in animal models of hemophilia A, like FVIII knockout mice in which one would expect an increased percentage of FVIII be found by antigen or activity assays in the circulation shortly (5 to 10 min.) after i.v. administration compared to the corresponding wild-type VWF, or reference polypeptide defined supra.

[0067] The in vivo recovery is preferably increased by at least 10%, more preferably by at least 20%, and even more preferably by at least 40% compared to FVIII complexed with wild-type VWF, or with the reference polypeptide defined supra.

Ratios

[0068] As described in more detail below, the polypeptide of the invention may be a monomer, a dimer, or a mixture thereof. Any molar ratios according to the invention refer to a ratio of the molar concentration of the monomeric subunit of the polypeptide of the invention, whether actually present as monomer or dimer. Ratios are formed either over the molar concentration of the co-administered FVIII or over the molar concentration of the endogenous VWF subunits. Any ratios of polypeptide of the invention over FVIII in this application refer to the amount of polypeptide of the invention to be administered (in mole) divided by the amount of FVIII to be administered (in mole), unless indicated otherwise. The endogenous VWF is the VWF which is naturally present in the plasma of the animal or human being to be dosed with the polypeptide of the invention and with the co-administered FVIII. It usually consists of a range of different oligomers of approximately 2 to 40 monomeric subunits of VWF. Unless indicated otherwise, any ratios of polypeptide of the invention over endogenous VWF in this application refer to the molar plasma concentration of polypeptide of the invention per kg body weight of the treated subject immediately after administration of the polypeptide of the invention, divided by the molar plasma concentration of endogenous VWF per kg body weight of the treated subject.

The molar plasma concentration of the polypeptide of the invention per kg body weight of the subject treated immediately after administration of the polypeptide of the invention is calculated assuming a dilution of the polypeptide of the invention administered directly after administration in a plasma volume of 40 ml/kg. The amount of the polypeptide of the invention immediately after administration when administered intravenously is assumed for the purposes of the invention to be identical to the amount administered.

[0069] Whilst the polypeptide of the present invention may be administered at any level an advantage may be achieved by administration at a level where the molar ratio of the polypeptide of the invention to the endogenous VWF is greater than 0.5. The concentration of endogenous VWF in the plasma of the subject to be treated can be determined by an ELISA or and activity assay, e.g. as described in the Examples. Typically, the concentration measured will be given in U/mL. This value can be converted into a molarity as described in the following.

[0070] Normal human plasma (NHP) contains VWF in a concentration of 1 U/mL or 100% by definition. This corresponds to a protein concentration of approximately 10µg/mL (Haberichter S.L. and Montgomery R.R., Structure and function of von Willebrand factor; in: Hemostasis and Thrombosis, eds. Marder, Aird, Bennett, Schulman and White, Lippincott Williams & Wilkins 2013, pp 197-207). Based on this VWF concentration in NHP and a molecular weight of the mature VWF monomer of approximately 267,500 Da including 18-19% of glycosylation a molar plasma concentration of the VWF monomer unit of approximately 37×10^{-9} Mol/L can be calculated for NHP.

[0071] For calculation of the molar concentrations of rat or rabbit VWF subunits in normal rat or rabbit plasma, respectively, a molecular weight of the monomeric subunit comparable to human VWF was used (267,500 Da) together with an assumed comparable specific activity (100 U/mg) and the measured endogenous VWF activities in rat or rabbit plasma (refer also to examples).

[0072] The concentration of VWF in the human population varies from about 60% to about 200% of VWF concentration in NHP. In certain embodiments of the invention the concentration of endogenous VWF is defined as the concentration in NHP. In other embodiments the concentration of endogenous VWF is determined in the subject to be treated, and the dose of the polypeptide is based on this individual value.

[0073] The molar ratio of the polypeptide of the invention administered to the endogenous VWF is preferably at least 2, or at least 3, or at least 4, or at least 5, or at least 6, or at least 7, or at least 8, or at least 9, or at least 10, more preferably at least 15, or at least 20, or at least 25, or at least 30, most preferably at least 40, or at least 50, or at least 75.

[0074] The molar ratio of the polypeptide of the invention to be administered to the endogenous VWF may range from 0.5 to 1,000, or from 1 to 500, or from 2 to 400, or from 3 to 300, or from 4 to 250, or from 5 to 200, or from 6 to 150, or from 7 to 140, or from 8 to 130, or from 9 to 120, or from 10 to 110. Preferably, the molar ratio of the polypeptide of the invention

administered to endogenous VWF ranges from 3 to 100, or from 4 to 90, or from 5 to 80, or from 6 to 75, or from 10 to 60.

[0075] The molar ratio of the polypeptide of the invention to be administered to FVIII to be administered is preferably at least 2, or at least 5, or at least 10, or at least 20, or at least 30, or at least 40, or at least 50, more preferably the ratio is greater than 50, or at least 75, at least 100, or greater than 100, or at least 200, most preferably at least 300, or at least 400, or at least 500.

[0076] The molar ratio of the polypeptide of the invention to be administered to FVIII to be administered may range from 2 to 10,000, or from 5 to 5,000, or from 10 to 4,000, or from 20 to 3,000, or from 30 to 2,000, or from 40 to 1,000. Preferably, the molar ratio of the polypeptide of the invention to be administered to FVIII to be administered ranges from 60 to 2,500, or from 110 to 2,000, or from 150 to 1,500, or from 200 to 1,000.

[0077] Table 1 summarizes various embodiments of the treatment in accordance with this invention. In a given embodiment, both requirements of column 2 and 3, respectively, must be fulfilled.

Table 2

Embodiment #	Molar ratio polypeptide of the invention : endogenous VWF	Molar ratio polypeptide of the invention : FVIII administered
1	at least 1	at least 2
2	at least 1	at least 5
3	at least 1	at least 10
4	at least 1	at least 40
5	at least 1	at least 50
6	at least 1	at least 80
7	at least 1	at least 100
6	at least 1	at least 150
7	at least 1	at least 250
8	at least 1	at least 400
9	at least 1	at least 800
10	at least 1	at least 1,000
11	at least 3	at least 2
12	at least 3	at least 5
13	at least 3	at least 10
14	at least 3	at least 40
15	at least 3	at least 50
16	at least 3	at least 80

Embodiment #	Molar ratio polypeptide of the invention : endogenous VWF	Molar ratio polypeptide of the invention : FVIII administered
17	at least 3	at least 100
18	at least 3	at least 150
19	at least 3	at least 250
20	at least 3	at least 400
21	at least 3	at least 800
22	at least 3	at least 1,000
23	at least 5	at least 2
24	at least 5	at least 5
25	at least 5	at least 10
26	at least 5	at least 40
27	at least 5	at least 50
28	at least 5	at least 80
29	at least 5	at least 100
30	at least 5	at least 150
31	at least 5	at least 250
32	at least 5	at least 400
33	at least 5	at least 800
34	at least 5	at least 1,000
35	at least 10	at least 2
36	at least 10	at least 5
37	at least 10	at least 10
38	at least 10	at least 40
39	at least 10	at least 50
40	at least 10	at least 80
41	at least 10	at least 100
42	at least 10	at least 150
43	at least 10	at least 250
44	at least 10	at least 400
45	at least 10	at least 800
46	at least 10	at least 1,000
47	at least 20	at least 2
48	at least 20	at least 5
49	at least 20	at least 10
50	at least 20	at least 40

Embodiment #	Molar ratio polypeptide of the invention : endogenous VWF	Molar ratio polypeptide of the invention : FVIII administered
51	at least 20	at least 50
52	at least 20	at least 80
53	at least 20	at least 100
54	at least 20	at least 150
55	at least 20	at least 250
56	at least 20	at least 400
57	at least 20	at least 800
58	at least 20	at least 1,000
59	at least 50	at least 2
60	at least 50	at least 5
61	at least 50	at least 10
62	at least 50	at least 40
63	at least 50	at least 50
64	at least 50	at least 80
65	at least 50	at least 100
66	at least 50	at least 150
67	at least 50	at least 250
68	at least 50	at least 400
69	at least 50	at least 800
70	at least 50	at least 1,000
71	at least 50	at least 2,000
72	at least 50	at least 4,000

[0078] Embodiments 1 to 72 shown in Table 2 can be combined with any other embodiment and aspect of the invention described herein. Further details of the treatment in accordance with the invention are described further below.

N-Glycans and Sialylation of the polypeptide of the invention

[0079] The polypeptide of the invention preferably comprises N-glycans, and at least 75%, preferably at least 85%, more preferably at least 90% of said N-glycans comprise, on average, at least one sialic acid moiety. In preferred embodiments, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, of said N-glycans comprise, on average, at least one sialic acid moiety. The inventors found that

polypeptides comprising highly sialylated VWF fragments not only have a prolonged half-life themselves, but are also capable to extend the half-life of co-administered FVIII. In other words, administration of the polypeptide of the invention leads to an extended half-life and/or to a reduced clearance of co-administered FVIII.

[0080] The polypeptide of the invention preferably comprises N-glycans, and at least 50% of the sialyl groups of the N-glycans of the glycoproteins are α -2,6-linked sialyl groups. In general, terminal sialyl groups can be attached to the galactose groups via a α -2,3- or via a α -2,6-linkage. Typically, N-glycans of the polypeptide of the invention comprise more α -2,6-linked sialyl groups than α -2,3-linked sialyl groups. Preferably, at least 60%, or at least 70%, or at least 80%, or at least 90% of the sialyl groups of the N-glycans are α -2,6-linked sialyl groups. These embodiments can be obtained by, e.g., co-expressing human α -2,6-sialyltransferase in mammalian cells.

[0081] In one embodiment, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, of the N-glycans of the polypeptide of the invention comprise at least one sialic acid group. In another embodiment, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, of the N-glycans of the polypeptide of the invention comprise at least one sialic acid group.

[0082] In another embodiment, less than 15%, less than 12%, less than 10%, or less than 8%, or less than 6%, or less than 5%, or less than 4%, or less than 3%, or less than 2% or even less than 1% of the N-glycans of the polypeptide of the invention are asialo-N-glycans, i.e. they are N-glycans lacking a sialic acid group. In another embodiment, less than 15%, less than 12%, less than 10%, or less than 8%, or less than 6%, or less than 5%, or less than 4%, or less than 3%, or less than 2% or even less than 1% of the N-glycans of the polypeptide of the invention are asialo-N-glycans, i.e. they do not have a sialic acid group.

[0083] The above-described embodiments can be combined with each other. Any percentages of N-glycans mentioned above, or any indications of the degree of sialylation, are to be understood as average percentages or degrees, i.e. they refer to a population of molecules, not to a single molecule. It is clear that the glycosylation or sialylation of the individual glycoprotein molecules within a population of glycoproteins will show some heterogeneity.

Dimers

[0084] It has further been found that the polypeptides of this invention may have a high proportion of dimers. The polypeptide of the invention is therefore preferably present as dimer. In one embodiment, at least 50%, or at least 60%, or at least 70% of the polypeptides are present as dimers. In another embodiment, the ratio dimer : monomer of the polypeptide of the invention is at least 1.5, preferably at least 2, more preferably at least 2.5 or at least 3. Most preferably all polypeptides of the invention are present as dimers. The use of dimers is favorable, as the dimer has an improved affinity to Factor VIII as compared to the monomer.

[0085] In one embodiment, the affinity of the polypeptide of the invention to Factor VIII is greater than that of human native VWF to the same Factor VIII molecule. The factor VIII affinity may refer to human native Factor VIII, or to the Factor VIII molecule characterized by SEQ ID NO:15.

[0086] It has been found that preparations of the polypeptide of this invention with a high proportion of dimers do have an increased affinity to Factor VIII. Such increased affinity to Factor VIII does lead to an enhanced stabilization of Factor VIII by the polypeptides of the present invention. Alternatively to or in combination with an increased dimer proportion also polypeptides in accordance with the invention with mutations within the Factor VIII binding domain which do increase the affinity to Factor VIII are preferred embodiments of the invention. Suitable mutations are disclosed, e.g., in WO 2013/120939 A1.

Preparation of the polypeptide

[0087] The nucleic acid encoding the polypeptide of the invention can be prepared according to methods known in the art. Based on the cDNA sequence of VWF (SEQ ID NO:3), recombinant DNA encoding the above-mentioned truncated VWF constructs or polypeptides of the invention can be designed and generated.

[0088] Even if the polypeptide which is secreted by the host cells does not comprise amino acids 1 to 763 of VWF, it is preferred that the nucleic acid (e.g. the DNA) encoding the intracellular precursor of the polypeptide comprises a nucleotide sequence encoding an amino acid sequence having a sequence identity of at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, to amino acids 23 to 763 or preferably to amino acids 1 to 763 of SEQ ID NO:2. Most preferably, the nucleic acid (e.g. the DNA) encoding the intracellular precursor of the polypeptide comprises a nucleotide sequence encoding amino acids 23 to 763 of SEQ ID NO:2, or amino acids 1 to 763 of SEQ ID NO:2.

[0089] Constructs in which the DNA contains the entire open reading frame inserted in the correct orientation into an expression plasmid may be used for protein expression. Typical expression vectors contain promoters that direct the synthesis of large amounts of mRNA corresponding to the inserted nucleic acid in the plasmid-bearing cells. They may also include an origin of replication sequence allowing for their autonomous replication within the host organism, and sequences that increase the efficiency with which the synthesized mRNA is translated. Stable long-term vectors may be maintained as freely replicating entities by using regulatory elements of, for example, viruses (e.g., the OriP sequences from the Epstein Barr Virus genome). Cell lines may also be produced that have integrated the vector into the genomic DNA, and in this manner the gene product is produced on a continuous basis.

[0090] Typically, the cells to be provided are obtained by introducing the nucleic acid encoding a polypeptide of the invention into mammalian host cells.

[0091] Any host cell susceptible to cell culture, and to expression of glycoproteins, may be utilized in accordance with the present invention. In certain embodiments, a host cell is mammalian. Non-limiting examples of mammalian cells that may be used in accordance with the present invention include BALB/c mouse myeloma line (NSO/ 1, ECACC No: 85110503); human retinoblasts (PER.C6 (CruCell, Leiden, The Netherlands)); monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., J. Gen Virol., 36:59, 1977); baby hamster kidney cells (BHK, ATCC CCL10); Chinese hamster ovary cells +/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77:4216, 1980); mouse sertoli cells (TM4, Mather, Biol. Reprod., 23:243 251, 1980); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1 587); human cervical carcinoma cells (HeLa, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (HepG2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., Annals NY. Acad. Sci., 383:44-68, 1982); MRC 5 cells; PS4 cells; human amniocyte cells (CAP); and a human hepatoma line (Hep G2). Preferably, the cell line is a rodent cell line, especially a hamster cell line such as CHO or BHK.

[0092] Methods suitable for introducing nucleic acids sufficient to achieve expression of a glycoprotein of interest into mammalian host cells are known in the art. See, for example, Gething et al., Nature, 293:620-625, 1981; Mantei et al., Nature, 281:40-46, 1979; Levinson et al. EP 117,060; and EP 117,058. For mammalian cells, common methods of introducing genetic material into mammalian cells include the calcium phosphate precipitation method of Graham and van der Erb (Virology, 52:456-457, 1978) or the lipofectamineTM (Gibco BRL) Method of Hawley-Nelson (Focus 15:73, 1993). General aspects of mammalian cell host system transformations have been described by Axel in US. Pat. No. 4,399,216. For various techniques for introducing genetic material into mammalian cells, see Keown et al., Methods in Enzymology, 1989, Keown et al., Methods in Enzymology, 185:527-537, 1990, and Mansour et al., Nature, 336:348-352, 1988.

[0093] The cells are cultured under conditions that allow expression of the polypeptide. The polypeptide can be recovered and purified using methods that are known to the skilled artisan.

Terminal half-life, MRT and Clearance

[0094] Another aspect of the invention is the use of a polypeptide as defined hereinabove for increasing the terminal half-life or mean residence time (MRT) or reducing the clearance of Factor VIII. For evaluation of the pharmacokinetic data a linear pharmacokinetics model (compound elimination via the central compartment) was applied. Accordingly, any pharmacokinetic parameters used herein are based on a linear pharmacokinetics model (compound elimination via the central compartment), unless indicated otherwise.

[0095] The "half-life" $T_{1/2}(t)$ at a certain time t is the time it takes to halve the plasma concentration $C(t)$ that is present at time t , i.e. $C[t + T_{1/2}(t)] = C(t)/2$. The "terminal half-life" is the limit of $T_{1/2}(t)$ when t tends to infinity.

[0096] The terminal half-life of administered FVIII is increased by at least 25%, preferably by at least 50%, more preferably by at least 75%, more preferably by at least 100%, most preferably by at least 150%, if an effective amount of the polypeptide of the present invention is co-administered, relative to administration of the FVIII alone. Another aspect of the invention is the use of a polypeptide as defined hereinabove for increasing the terminal half-life of Factor VIII.

[0097] The term "MRT", as used herein, means the average time a drug molecule (e.g. the polypeptide of the invention or a FVIII) resides in the body. In a linear pharmacokinetic system with constant clearance MRT can be calculated as the area under the first moment curve (AUMC) divided by the area under the plasma concentration-time curve (AUC). The first moment curve is time multiplied by plasma concentration at that time.

[0098] The MRT of administered FVIII is increased by at least 25%, preferably by at least 50%, more preferably by at least 75%, more preferably by at least 100%, most preferably by at least 150%, if an effective amount of the polypeptide of the present invention is co-administered, relative to administration of the FVIII alone. Another aspect of the invention is the use of a polypeptide as defined hereinabove for increasing the terminal half-life or mean residence time (MRT) or reducing the clearance of Factor VIII.

[0099] The term "clearance", as used herein, refers to the rate at which plasma is cleared of drug. Specifically, it is the current elimination rate of a drug divided by its current plasma concentration. In a linear pharmacokinetic system after a single intravenous administration the clearance can be calculated as the ratio of dose over the area under the plasma concentration-time curve (AUC), provided the clearance is constant. The lower the clearance the longer it takes until the plasma is cleared of the drug.

[0100] The clearance of administered FVIII is reduced by at least 10%, preferably by at least 25%, more preferably by at least 50%, more preferably by at least 60%, most preferably by at least 70%, if an effective amount of the polypeptide of the present invention is co-administered, relative to administration of the FVIII alone.

[0101] The invention further relates to a method of increasing the MRT or half-life, or to a method of reducing the clearance of Factor VIII in vivo, comprising administering to a subject an effective amount of a polypeptide as defined hereinabove.

[0102] A further aspect of this invention is the polypeptide of the invention for treating a blood coagulation disorder, comprising administering to a patient in need thereof an effective amount of a polypeptide as defined hereinabove.

[0103] A further aspect is the use of a polypeptide as defined hereinabove for reducing the

frequency of administration of FVIII in a treatment of hemophilia A. The frequency of intravenous or subcutaneous administration of FVIII may be reduced to twice per week. Alternatively, the frequency of intravenous or subcutaneous administration of FVIII may be reduced to once per week, or even lower, e.g. to once per 10 days or once per 14 days. The FVIII may be administered twice weekly, every 5 days, once weekly, every 10 days, every two weeks, every three weeks, every four weeks or once a month, or in any range between any two of the foregoing values, for example from every four days to every month, from every 10 days to every two weeks, or from two to three times a week, etc.

[0104] Another aspect is the use of a polypeptide as defined hereinabove for reducing the dose of FVIII to be administered in a treatment of hemophilia A.

Treatment of coagulation disorder

[0105] The polypeptides of the invention are useful for treating coagulation disorders including hemophilia A. The term "hemophilia A" refers to a deficiency in functional coagulation FVIII, which is usually inherited.

[0106] Treatment of a disease encompasses the treatment of patients already diagnosed as having any form of the disease at any clinical stage or manifestation; the delay of the onset or evolution or aggravation or deterioration of the symptoms or signs of the disease; and/or preventing and/or reducing the severity of the disease.

[0107] A "subject" or "patient" to whom a polypeptide of the invention is administered preferably is a human. In certain aspects, the human is a pediatric patient. In other aspects, the human is an adult patient.

[0108] Compositions comprising a polypeptide of the invention and, optionally FVIII, are described herein. The compositions typically are supplied as part of a sterile, pharmaceutical composition that includes a pharmaceutically acceptable carrier. This composition can be in any suitable form (depending upon the desired method of administering it to a patient).

[0109] The term "Factor VIII" and "FVIII" are used interchangeably herein and encompass both plasma derived FVIII and recombinant FVIII. Recombinant FVIII encompasses without limitation full-length FVIII as well as two-chain B-domain deleted or truncated variants as well as single-chain B-domain deleted or truncated variants for example those described in WO 2004/067566 and other FVIII variants with mutations outside the B-domain but having the biological activity of FVIII.

[0110] The polypeptide of the invention can be administered to a patient by a variety of routes such as orally, transdermally, subcutaneously, intranasally, intravenously, intraperitoneally, intramuscularly, topically or locally. The most suitable route for administration in any given case will depend on the particular polypeptide, the subject, and the nature and severity of the

disease and the physical condition of the subject. Typically, a polypeptide of the invention will be administered intravenously.

[0111] The polypeptide and the FVIII are preferably administered intravenously or subcutaneously.

[0112] In a first embodiment, both the polypeptide and the FVIII are administered intravenously. In a second embodiment, both the polypeptide and the FVIII are administered subcutaneously.

[0113] In another embodiment, the FVIII is administered intravenously, and the polypeptide is administered via a different route. In further embodiments, the polypeptide is administered subcutaneously, and the FVIII is administered via a different route. For example, the polypeptide may be administered subcutaneously, and the FVIII may be administered intravenously.

[0114] In further embodiments, the FVIII is administered subcutaneously, and the polypeptide is administered via a different route. In further embodiments, the polypeptide is administered intravenously, and the FVIII is administered via a different route. For example, the polypeptide may be administered intravenously, and the FVIII may be administered subcutaneously.

[0115] Determination of the total number of doses, and length of treatment with a polypeptide of the invention is well within the capabilities of those skilled in the art. The dosage of the polypeptide of the invention to be administered depends on the concentrations of the FVIII to be administered, the concentration of endogenous VWF in the patient to be treated, or both. An effective dosage based on the ratios defined by the inventors of this application can be determined by the skilled person, taking into account the molecular weight of the polypeptide of the invention. Typical dosages for FVIII may range from about 20 U/kg body weight to about 100 U/kg body weight.

[0116] In accordance with this invention, the patient being treated with the polypeptide of the invention is also treated with blood coagulation Factor VIII. The polypeptide of the invention and the Factor VIII may be administered simultaneously or in a sequential fashion both modes of administration being encompassed by the term "combination therapy" and "co-administration". The polypeptide of the invention and the Factor VIII may be administered as a mixture, i.e. within the same composition, or separately, i.e. as separate compositions.

[0117] The concentration of Factor VIII in the composition used is typically in the range of 10-10,000 IU/mL. In different embodiments, the concentration of FVIII in the compositions of the invention is in the range of 10-8,000 IU/mL, or 10-5,000 IU/mL, or 20-3,000 IU/mL, or 50-1,500 IU/mL, or 3,000 IU/mL, or 2,500 IU/mL, or 2,000 IU/mL, or 1,500 IU/mL, or 1,200 IU/mL, or 1,000 IU/mL, or 800 IU/mL, or 750 IU/mL, or 600 IU/mL, or 500 IU/mL, or 400 IU/mL, or 300 IU/mL, or 250 IU/mL, or 200 IU/mL, or 150 IU/mL, or 125 IU/mL, or 100 IU/mL, or 62.5 IU/mL, or 50 IU/mL, provided the requirements regarding the ratio with respect to the VWF

polypeptide of the invention as defined herein are fulfilled.

[0118] "International Unit," or "IU," is a unit of measurement of the blood coagulation activity (potency) of FVIII as measured by a FVIII activity assay such as a one stage clotting assay or a chromogenic substrate FVIII activity assay using a standard calibrated against an international standard preparation calibrated in "IU". One stage clotting assays are known to the art, such as that described in N Lee, Martin L, et al., An Effect of Predilution on Potency Assays of FVIII Concentrates, Thrombosis Research (Pergamon Press Ltd.) 30, 511 519 (1983). Principle of the one stage assay: The test is executed as a modified version of the activated Partial Thromboplastin Time (aPTT)-assay: Incubation of plasma with phospholipids and a surface activator leads to the activation of factors of the intrinsic coagulation system. Addition of calcium ions triggers the coagulation cascade. The time to formation of a measurable fibrin clot is determined. The assay is executed in the presence of Factor VIII deficient plasma. The coagulation capability of the deficient plasma is restored by Coagulation Factor VIII included in the sample to be tested. The shortening of coagulation time is proportional to the amount of Factor VIII present in the sample. The activity of Coagulation Factor VIII is quantified by direct comparison to a standard preparation with a known activity of Factor VIII in International Units.

[0119] Another standard assay is a chromogenic substrate assay. Chromogenic substrate assays may be purchased commercially, such as the coamatic FVIII test kit (Chromogenix-Instrumentation Laboratory SpA V. le Monza 338 - 20128 Milano, Italy). Principle of the chromogenic assay: In the presence of calcium and phospholipid, Factor X is activated by Factor IXa to Factor Xa. This reaction is stimulated by Factor VIIIa as cofactor. FVIIIa is formed by low amounts of thrombin in the reaction mixture from FVIII in the sample to be measured. When using the optimum concentrations of Ca^{2+} , phospholipid and Factor IXa and an excess quantity of Factor X, activation of Factor X is proportional to the potency of Factor VIII. Activated Factor X releases the chromophore pNA from the chromogenic substrate S-2765. The release of pNA, measured at 405 nm, is therefore proportional to the amount of FXa formed, and, therefore, also to the Factor VIII activity of the sample.

Pharmaceutical compositions

[0120] Therapeutic formulations of the polypeptide of the invention suitable in the methods described herein can be prepared for storage as lyophilized formulations or aqueous solutions by mixing the polypeptide having the desired degree of purity with optional pharmaceutically-acceptable carriers, excipients or stabilizers typically employed in the art (all of which are referred to herein as "carriers"), i.e., buffering agents, stabilizing agents, preservatives, isotonicifiers, non-ionic detergents, antioxidants, and other miscellaneous additives. See, Remington's Pharmaceutical Sciences, 16th edition (Osol, ed. 1980). Such additives must be nontoxic to the recipients at the dosages and concentrations employed.

[0121] Buffering agents help to maintain the pH in the range which approximates physiological conditions. They can be present at concentration ranging from about 2 mM to about 50 mM.

Suitable buffering agents include both organic and inorganic acids and salts thereof such as citrate buffers (e.g., monosodium citrate-disodium citrate mixture, citric acid-trisodium citrate mixture, citric acid-monosodium citrate mixture, etc.), succinate buffers (e.g., succinic acid-monosodium succinate mixture, succinic acid-sodium hydroxide mixture, succinic acid-disodium succinate mixture, etc.), tartrate buffers (e.g., tartaric acid-sodium tartrate mixture, tartaric acid-potassium tartrate mixture, tartaric acid-sodium hydroxide mixture, etc.), fumarate buffers (e.g., fumaric acid-monosodium fumarate mixture, fumaric acid-disodium fumarate mixture, monosodium fumarate-disodium fumarate mixture, etc.), gluconate buffers (e.g., gluconic acid-sodium glyconate mixture, gluconic acid-sodium hydroxide mixture, gluconic acid-potassium gluconate mixture, etc.), oxalate buffer (e.g., oxalic acid-sodium oxalate mixture, oxalic acid-sodium hydroxide mixture, oxalic acid-potassium oxalate mixture, etc), lactate buffers (e.g., lactic acid-sodium lactate mixture, lactic acid-sodium hydroxide mixture, lactic acid-potassium lactate mixture, etc.) and acetate buffers (e.g., acetic acid-sodium acetate mixture, acetic acid-sodium hydroxide mixture, etc.). Additionally, phosphate buffers, histidine buffers and trimethylamine salts such as Tris can be used.

[0122] Preservatives can be added to retard microbial growth, and can be added in amounts ranging from 0.2%- 1% (w/v). Suitable preservatives include phenol, benzyl alcohol, meta-cresol, methyl paraben, propyl paraben, octadecyldimethylbenzyl ammonium chloride, benzalconium halides (e.g., chloride, bromide, and iodide), hexamethonium chloride, and alkyl parabens such as methyl or propyl paraben, catechol, resorcinol, cyclohexanol, and 3-pentanol. Isotonicifiers sometimes known as "stabilizers" can be added to ensure isotonicity of liquid compositions and include polyhydric sugar alcohols, preferably trihydric or higher sugar alcohols, such as glycerin, erythritol, arabitol, xylitol, sorbitol and mannitol. Stabilizers refer to a broad category of excipients which can range in function from a bulking agent to an additive which solubilizes the therapeutic agent or helps to prevent denaturation or adherence to the container wall. Typical stabilizers can be polyhydric sugar alcohols (enumerated above); amino acids such as arginine, lysine, glycine, glutamine, asparagine, histidine, alanine, ornithine, L-leucine, 2-phenylalanine, glutamic acid, threonine, etc., organic sugars or sugar alcohols, such as lactose, trehalose, stachyose, mannitol, sorbitol, xylitol, ribitol, myoinositol, galactitol, glycerol and the like, including cyclitols such as inositol; polyethylene glycol; amino acid polymers; sulfur containing reducing agents, such as urea, glutathione, thiocetic acid, sodium thioglycolate, thioglycerol, α -monothioglycerol and sodium thio sulfate; low molecular weight polypeptides (e.g., peptides of 10 residues or fewer); proteins such as human serum albumin, bovine serum albumin, gelatin or immunoglobulins; hydrophilic polymers, such as polyvinylpyrrolidone monosaccharides, such as xylose, mannose, fructose, glucose; disaccharides such as lactose, maltose, sucrose and trisaccharides such as raffinose; and polysaccharides such as dextran. Stabilizers can be present in the range from 0.1 to 10,000 weights per part of weight active protein.

[0123] Non-ionic surfactants or detergents (also known as "wetting agents") can be added to help solubilize the therapeutic agent as well as to protect the therapeutic protein against agitation-induced aggregation, which also permits the formulation to be exposed to shear surface stressed without causing denaturation of the protein. Suitable non-ionic surfactants

include polysorbates (20, 80, etc.), polyoxamers (184, 188 etc.), Pluronic polyols, polyoxyethylene sorbitan monoethers (TWEEN®-20, TWEEN®-80, etc.). Non-ionic surfactants can be present in a range of about 0.05 mg/ml to about 1.0 mg/ml, or in a range of about 0.07 mg/ml to about 0.2 mg/ml.

[0124] Additional miscellaneous excipients include bulking agents (e.g., starch), chelating agents (e.g., EDTA), antioxidants (e.g., ascorbic acid, methionine, vitamin E), and cosolvents.

[0125] The formulation herein can also contain a second therapeutic agent in addition to a polypeptide of the invention. Examples of suitable second therapeutic agents are provided below.

[0126] The dosing schedule can vary from once a month to daily depending on a number of clinical factors, including the type of disease, severity of disease, and the patient's sensitivity to the polypeptide of the invention. In specific embodiments, a polypeptide of the invention is administered, twice weekly, every 5 days, once weekly, every 10 days, every two weeks, every three weeks, every four weeks or once a month, or in any range between any two of the foregoing values, for example from every four weeks to every month, from every 10 days to every two weeks, or from two to three times a week, etc.

[0127] The dosage of a polypeptide of the invention to be administered will vary according to the particular polypeptide, the subject, and the nature and severity of the disease, the physical condition of the subject, the therapeutic regimen (e.g., whether a second therapeutic agent is used), and the selected route of administration; the appropriate dosage can be readily determined by a person skilled in the art.

[0128] It will be recognized by one of skill in the art that the optimal quantity and spacing of individual dosages of a polypeptide of the invention will be determined by the nature and extent of the condition being treated, the form, route and site of administration, and the age and condition of the particular subject being treated, and that a physician will ultimately determine appropriate dosages to be used. This dosage can be repeated as often as appropriate. If side effects develop the amount and/or frequency of the dosage can be altered or reduced, in accordance with normal clinical practice.

[0129] The binding affinity of the polypeptide of the present invention to FVIII is higher than that of a reference polypeptide which has the same amino acid sequence except for the modification(s) in SEQ ID NO:3.

[0130] The binding affinity of a VWF molecule to a Factor VIII molecule can be determined by a binding assay used in the art. For example, the VWF molecule may be immobilized on a solid support, increasing concentrations of Factor VIII are applied, incubated for a certain period of time, and after washing, bound Factor VIII is determined with a chromogenic assay. The affinity constant or dissociation constant may then be determined by Scatchard analysis or another suitable method. A method of determining the affinity of binding of human Factor VIII to von

Willebrand Factor are described in Vlot et al. (1995), Blood, Volume 85, Number 11, 3150-3157.

[0131] Any indication herein of affinity, including dissociation constants, preferably refers to the binding of the modified VWF of the invention, or of the polypeptide of the invention to FVIII. The amino acid sequence of single chain of FVIII is shown in SEQ ID NO:15.

[0132] As the interaction of VWF with FVIII typically has a high on-rate, changes in the dissociation constant is largely dependent on changes in the off-rate. Accordingly the main focus in increasing the association of VWF with FVIII involves efforts to decrease the off-rate between FVIII and VWF. Preferably the off-rate of the modified VWF and FVIII in comparison to wild type VWF and FVIII is at least two fold lower, more preferably at least 5 fold lower, preferably at least 10 fold lower and more preferably at least 20 fold lower.

[0133] The dissociation constant of the complex consisting of VWF and FVIII is preferably 0.2 nmol/L or less, more preferably 0.175 nmol/L or less, more preferably 0.15 nmol/L or less, more preferably 0.125 nmol/L or less, more preferably 0.1 nmol/L or less, more preferably 0.05 nmol/L or less, most preferably 0.01 nmol/L or less.

[0134] The dissociation constant K_D of a complex of the polypeptide of the invention and the Factor VIII of SEQ ID NO:15 is typically less than 90% of the dissociation constant K_D of a complex of the reference polypeptide (e.g. the polypeptide of SEQ ID NO:4) and the Factor VIII of SEQ ID NO:15. The dissociation constant K_D of a complex of the polypeptide of the invention and the Factor VIII of SEQ ID NO:14 is preferably less than 75%, more preferably less than 50%, more preferably less than 25%, more preferably less than 10%, more preferably less than 5%, of the dissociation constant K_D of a complex of the reference polypeptide (e.g. the polypeptide of SEQ ID NO:3) and the Factor VIII of SEQ ID NO:15.

[0135] The reference polypeptide is a polypeptide the amino acid sequence of which is identical to that of the polypeptide of the present invention except for the mutation within the D'-D3 domains of VWF. That is, the reference polypeptide preferably has an amino acid sequence identical to that of the polypeptide of the present invention, with the proviso that the D'-D3 domains in the reference polypeptide consist of the amino acid sequence as shown in SEQ ID NO:3. In other words, the only difference in sequence between the polypeptide of the invention and the reference polypeptide lies in the amino acid sequence of the D'-D3 domains. The reference polypeptide has preferably been prepared under the same conditions as the polypeptide of the invention.

Polynucleotides

[0136] The invention further relates to a polynucleotide encoding a modified VWF or a polypeptide comprising said modified VWF, as described in this application. The term "polynucleotide(s)" generally refers to any polyribonucleotide or polydeoxyribonucleotide that

may be unmodified RNA or DNA or modified RNA or DNA. The polynucleotide may be single- or double-stranded DNA, single or double-stranded RNA. As used herein, the term "polynucleotide(s)" also includes DNAs or RNAs that comprise one or more modified bases and/or unusual bases, such as inosine. It will be appreciated that a variety of modifications may be made to DNA and RNA that serve many useful purposes known to those of skill in the art. The term "polynucleotide(s)" as it is employed herein embraces such chemically, enzymatically or metabolically modified forms of polynucleotides, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including, for example, simple and complex cells.

[0137] The skilled person will understand that, due to the degeneracy of the genetic code, a given polypeptide can be encoded by different polynucleotides. These "variants" are encompassed by this invention.

[0138] Preferably, the polynucleotide of the invention is an isolated polynucleotide. The term "isolated" polynucleotide refers to a polynucleotide that is substantially free from other nucleic acid sequences, such as and not limited to other chromosomal and extrachromosomal DNA and RNA. Isolated polynucleotides may be purified from a host cell. Conventional nucleic acid purification methods known to skilled artisans may be used to obtain isolated polynucleotides. The term also includes recombinant polynucleotides and chemically synthesized polynucleotides.

[0139] The invention further relates to a group of polynucleotides which together encode the modified VWF of the invention, or the polypeptide of the invention comprising the modified VWF. A first polynucleotide in the group may encode the N-terminal part of the modified VWF, and a second polynucleotide may encode the C-terminal part of the modified VWF.

[0140] Yet another aspect of the invention is a plasmid or vector comprising a polynucleotide according to the invention. Preferably, the plasmid or vector is an expression vector. In a particular embodiment, the vector is a transfer vector for use in human gene therapy.

[0141] The invention also relates to a group of plasmids or vectors that comprise the above group of polynucleotides. A first plasmid or vector may contain said first polynucleotide, and a second plasmid or vector may contain said second polynucleotide. Alternatively, both coding sequences are cloned into one expression vector either using two separate promoter sequences or one promoter and an internal ribosome entry site (IRES) element which may be used for example to direct the expression of furin to enhance the generation of mature VWF.

[0142] Still another aspect of the invention is a host cell comprising a polynucleotide, a plasmid or vector of the invention, or a group of polynucleotides or a group of plasmids or vectors as described herein.

[0143] The host cells of the invention may be employed in a method of producing a modified VWF or a polypeptide comprising said modified VWF, which is part of this invention. The method comprises:

1. (a) culturing host cells of the invention under conditions such that the desired modified protein is expressed; and
2. (b) optionally recovering the desired modified protein from the host cells or from the culture medium.

[0144] It is preferred to purify the modified VWF of the present invention, or the polypeptide comprising the modified VWF to $\geq 80\%$ purity, more preferably $\geq 95\%$ purity, and particularly preferred is a pharmaceutically pure state that is greater than 99.9% pure with respect to contaminating macromolecules, particularly other proteins and nucleic acids, and free of infectious and pyrogenic agents. Preferably, an isolated or purified modified VWF of the invention or polypeptide of the invention is substantially free of other, non-related polypeptides.

[0145] The various products of the invention are useful as medicaments. Accordingly, the invention relates to a pharmaceutical composition comprising a modified VWF or a polypeptide comprising said modified VWF as described herein, a polynucleotide of the invention, or a plasmid or vector of the invention.

[0146] The invention also concerns a method of treating an individual suffering from a blood coagulation disorder such as hemophilia A or B or VWD. The references to methods of treatment of this description are to be interpreted as references to the compounds, pharmaceutical compositions and medicaments of the present invention for use in a method for treatment of the human (or animal) body by therapy (or for diagnosis).

[0147] The method comprises administering to said individual an efficient amount of (i) FVIII and of the modified VWF or the polypeptide comprising the modified VWF or (ii) of the complex of FVIII with modified VWF or (iii) of the complex of FVIII with the polypeptide comprising modified VWF as described herein. In another embodiment, the method comprises administering to the individual an efficient amount of a polynucleotide of the invention or of a plasmid or vector of the invention. Alternatively, the method may comprise administering to the individual an efficient amount of the host cells of the invention described herein.

Expression of the modified polypeptides

[0148] The production of recombinant mutant proteins at high levels in suitable host cells requires the assembly of the above-mentioned modified polynucleotides, typically cDNA, into efficient transcriptional units together with suitable regulatory elements in a recombinant expression vector that can be propagated in various expression systems according to methods known to those skilled in the art. Efficient transcriptional regulatory elements could be derived from viruses having animal cells as their natural hosts or from the chromosomal DNA of animal cells. Preferably, promoter-enhancer combinations derived from the Simian Virus 40, adenovirus, BK polyoma virus, human cytomegalovirus, or the long terminal repeat of Rous

sarcoma virus, or promoter-enhancer combinations including strongly constitutively transcribed genes in animal cells like beta-actin or GRP78 can be used. In order to achieve stable high levels of mRNA transcribed from the cDNAs, the transcriptional unit should contain in its 3'-proximal part a DNA region encoding a transcriptional termination-polyadenylation sequence. Preferably, this sequence is derived from the Simian Virus 40 early transcriptional region, the rabbit beta-globin gene, or the human tissue plasminogen activator gene.

[0149] The cDNAs are then integrated into the genome of a suitable host cell line for expression of the modified FVIII and/or VWF proteins. Preferably this cell line should be an animal cell-line of vertebrate origin in order to ensure correct folding, disulfide bond formation, asparagine-linked glycosylation and other post-translational modifications as well as secretion into the cultivation medium. Examples on other post-translational modifications are tyrosine O-sulfation and proteolytic processing of the nascent polypeptide chain. Examples of cell lines that can be used are monkey COS-cells, mouse L-cells, mouse C127-cells, hamster BHK-21 cells, human embryonic kidney 293 cells, and hamster CHO-cells.

[0150] The recombinant expression vector encoding the corresponding cDNAs can be introduced into an animal cell line in several different ways. For instance, recombinant expression vectors can be created from vectors based on different animal viruses. Examples of these are vectors based on baculovirus, vaccinia virus, adenovirus, and preferably bovine papilloma virus.

[0151] The transcription units encoding the corresponding DNA's can also be introduced into animal cells together with another recombinant gene which may function as a dominant selectable marker in these cells in order to facilitate the isolation of specific cell clones which have integrated the recombinant DNA into their genome. Examples of this type of dominant selectable marker genes are Tn5 amino glycoside phosphotransferase, conferring resistance to gentamycin (G418), hygromycin phosphotransferase, conferring resistance to hygromycin, and puromycin acetyl transferase, conferring resistance to puromycin. The recombinant expression vector encoding such a selectable marker can reside either on the same vector as the one encoding the cDNA of the desired protein, or it can be encoded on a separate vector which is simultaneously introduced and integrated to the genome of the host cell, frequently resulting in a tight physical linkage between the different transcription units.

[0152] Other types of selectable marker genes which can be used together with the cDNA of the desired protein are based on various transcription units encoding dihydrofolate reductase (dhfr). After introduction of this type of gene into cells lacking endogenous dhfr-activity, preferentially CHO-cells (DUKX-B11, DG-44), it will enable these to grow in media lacking nucleosides. An example of such a medium is Ham's F12 without hypoxanthine, thymidine, and glycine. These dhfr-genes can be introduced together with the FVIII cDNA transcriptional units into CHO-cells of the above type, either linked on the same vector or on different vectors, thus creating dhfr-positive cell lines producing recombinant protein.

[0153] If the above cell lines are grown in the presence of the cytotoxic dhfr-inhibitor

methotrexate, new cell lines resistant to methotrexate will emerge. These cell lines may produce recombinant protein at an increased rate due to the amplified number of linked dhfr and the desired protein's transcriptional units. When propagating these cell lines in increasing concentrations of methotrexate (1-10000 nM), new cell lines can be obtained which produce the desired protein at very high rate.

[0154] The above cell lines producing the desired protein can be grown on a large scale, either in suspension culture or on various solid supports. Examples of these supports are micro carriers based on dextran or collagen matrices, or solid supports in the form of hollow fibres or various ceramic materials. When grown in cell suspension culture or on micro carriers the culture of the above cell lines can be performed either as a bath culture or as a perfusion culture with continuous production of conditioned medium over extended periods of time. Thus, according to the present invention, the above cell lines are well suited for the development of an industrial process for the production of the desired recombinant mutant proteins

Purification and Formulation

[0155] The recombinant modified VWF protein, which accumulates in the medium of secreting cells of the above types, can be concentrated and purified by a variety of biochemical and chromatographic methods, including methods utilizing differences in size, charge, hydrophobicity, solubility, specific affinity, etc. between the desired protein and other substances in the cell cultivation medium.

[0156] An example of such purification is the adsorption of the recombinant mutant protein to a monoclonal antibody, directed to e.g. a HLEP, preferably human albumin, or directed to the respective coagulation factor, which is immobilised on a solid support. After adsorption of the modified VWF to the support, washing and desorption, the protein can be further purified by a variety of chromatographic techniques based on the above properties.

[0157] The order of the purification steps is chosen e.g. according to capacity and selectivity of the steps, stability of the support or other aspects. Preferred purification steps include but are not limited to ion exchange chromatography steps, immune affinity chromatography steps, affinity chromatography steps, hydrophobic interaction chromatography steps, dye chromatography steps, hydroxyapatite chromatography steps, multimodal chromatography steps, and size exclusion chromatography steps.

[0158] In order to minimize the theoretical risk of virus contaminations, additional steps may be included in the process that provide effective inactivation or elimination of viruses. Such steps e.g. are heat treatment in the liquid or solid state, treatment with solvents and/or detergents, radiation in the visible or UV spectrum, gamma-radiation or nanofiltration.

[0159] The modified polynucleotides (e.g. DNA) of this invention may also be integrated into a transfer vector for use in the human gene therapy.

[0160] The various embodiments described herein may be combined with each other. The present invention will be further described in more detail in the following examples thereof. This description of specific embodiments of the invention will be made in conjunction with the appended figures.

[0161] The modified VWF as described in this invention can be formulated into pharmaceutical preparations for therapeutic use. The purified protein may be dissolved in conventional physiologically compatible aqueous buffer solutions to which there may be added, optionally, pharmaceutical excipients to provide pharmaceutical preparations.

[0162] Such pharmaceutical carriers and excipients as well as suitable pharmaceutical formulations are well known in the art (see for example "Pharmaceutical Formulation Development of Peptides and Proteins", Frokjaer et al., Taylor & Francis (2000) or "Handbook of Pharmaceutical Excipients", 3rd edition, Kibbe et al., Pharmaceutical Press (2000)). Standard pharmaceutical formulation techniques are well known to persons skilled in the art (see, e.g., 2005 Physicians' Desk Reference®, Thomson Healthcare: Montvale, NJ, 2004; Remington: The Science and Practice of Pharmacy, 20th ed., Gennaro et al., Eds. Lippincott Williams & Wilkins: Philadelphia, PA, 2000). In particular, the pharmaceutical composition comprising the polypeptide variant of the invention may be formulated in lyophilized or stable liquid form. The polypeptide variant may be lyophilized by a variety of procedures known in the art. Lyophilized formulations are reconstituted prior to use by the addition of one or more pharmaceutically acceptable diluents such as sterile water for injection or sterile physiological saline solution.

[0163] Formulations of the composition are delivered to the individual by any pharmaceutically suitable means of administration. Various delivery systems are known and can be used to administer the composition by any convenient route. Preferentially, the compositions of the invention are administered systemically. For systemic use, the proteins of the invention are formulated for parenteral (e.g. intravenous, subcutaneous, intramuscular, intraperitoneal, intracerebral, intrapulmonary, intranasal or transdermal) or enteral (e.g., oral, vaginal or rectal) delivery according to conventional methods. The most preferential routes of administration are intravenous and subcutaneous administration. The formulations can be administered continuously by infusion or by bolus injection. Some formulations encompass slow release systems.

[0164] The proteins of the present invention are administered to patients in a therapeutically effective dose, meaning a dose that is sufficient to produce the desired effects, preventing or lessening the severity or spread of the condition or indication being treated without reaching a dose which produces intolerable adverse side effects. The exact dose depends on many factors as e.g. the indication, formulation, and mode of administration and has to be determined in preclinical and clinical trials for each respective indication.

[0165] The pharmaceutical composition of the invention may be administered alone or in

conjunction with other therapeutic agents. These agents may be incorporated as part of the same pharmaceutical. One example of such an agent is the combination of modified VWF with FVIII.

[0166] "N-linked glycans" are oligosaccharides that are covalently linked to asparagine residues of a polypeptide. Terminal galactoses on such N-linked glycans may be modified by the attachment of an α -2,3- or an α -2,6-linked sialic acid.

[0167] The term "sialic acid" refers to the N- or O-substituted derivatives of neuraminic acid usually found as terminal monosaccharides of animal oligosaccharides (for review, see Varkis (1992) Glycobiology vol. 2 no. 1 pp. 25-40). The most common sialic acid is N-acetyl neuraminic acid. An "increased sialylation" means that at least 85% of the N-glycans of the glycoprotein comprise, on average, at least one sialic acid moiety. By way of non-limiting example an "increased sialylation of at least 85%" is determined as in Example 6 of the present invention, i.e. by enzymatically cleaving all N-glycans from a given glycoprotein of interest and then determining the amount of cleaved N-glycans with no sialic acids ("asialo N-glycans") and the total amount of all cleaved N-glycans. A "sialylation of at least 85%" corresponds then to an amount of 15% of asialo N-glycans or less of the total amount of all cleaved N-glycans.

[0168] In a first step, the methods of the invention comprise the step of providing cells comprising a nucleic acid encoding a polypeptide comprising a truncated von Willebrand Factor (VWF).

[0169] As described in more detail below, the method of the invention comprises providing cells comprising a nucleic acid encoding the polypeptide comprising the truncated VWF. The nucleic acid is introduced into suitable host cells by techniques that are known per se.

Culturing the Cells

[0170] In an embodiment the invention comprises culturing the cells at a temperature of less than 36.0°C. This method comprises culturing the cells under conditions that allow expression of the polypeptide.

[0171] The basal medium chosen for culturing the host cell line is not critical to the present invention and may be any one of, or combination of, those known to the art which are suitable for culturing mammalian cells. Media such as Dulbecco's Modified Eagle Medium, Ham's F-12 Medium, Eagle's Minimal Essential Medium and RPMI-1640 Medium and the like are commercially available. The addition of growth factors such as recombinant insulin is optional. In one embodiment, the medium is "protein-free" in the sense that it is either completely free of any protein or at least are free of any protein that is not recombinantly produced. Human serum albumin may be used as a serum-free culture supplement for the production of the polypeptide. Preferably, the medium contains a protease inhibitor, such as a serine protease inhibitor, which is suitable for tissue culture and which is of synthetic or vegetable origin.

[0172] Generally, the present invention may be used with any cell culture method that is amenable to the expression of polypeptides. For example, cells may be grown in batch or fed-batch cultures, where the culture is terminated after sufficient expression of the polypeptide, after which the expressed polypeptide is harvested. Alternatively, cells may be grown in continuous cultures (e.g. perfusion cultures), where the culture is not terminated and new nutrients and other components are periodically or continuously added to the culture, during which the expressed polypeptide is harvested periodically or continuously. The latter embodiment is preferred if the method comprises a temperature shift as described herein below. The culture can be any conventional type of culture, such as batch, fed-batch or continuous, but is preferably continuous. Suitable continuous cultures include perfusion culture.

[0173] Cells may be grown in any convenient volume chosen by the practitioner. For example, cells may be grown in small scale reaction vessels ranging in volume from a few milliliters to several liters. Alternatively, cells may be grown in large scale commercial bioreactors ranging in volume from approximately at least 1 liter to 10, 100, 250, 500, 1000, 2500, 5000, 8000, 10,000, 12,000 liters or more, or any volume in between. The culture is typically carried out in a bioreactor, which is usually a stainless steel, glass or plastic vessel of 1 (one) to 10000 (ten thousand) litres capacity, for example 5, 10, 50, 100, 1000, 2500, 5000 or 8000 litres. The vessel is usually rigid but flexible plastic bags can be used, particularly for smaller volumes. These are generally of the 'single use' type.

[0174] Mammalian cells such as CHO and BHK cells are generally cultured as suspension cultures. That is to say, the cells are suspended in the medium, rather than adhering to a solid support. The cells may alternatively be immobilized on a carrier, in particular on a microcarrier. Porous carriers, such as Cytoline[®], Cytopore[®] or Cytodex[®], may be particularly suitable.

[0175] To obtain a high sialylation, the cells (e.g. CHO cells) are preferably cultured at a decreased temperature, e.g. at less than 36.0°C. "Decreased temperature" refers to a temperature that is lower than the optimum temperature or normal temperature for growth of the respective cell line. For most mammalian cells the normal temperature is 37°C. It is therefore preferred according to the invention that the cells (e.g. CHO cells) are cultured at a decreased temperature of 30.0 to 36.0°C, 30.5 to 35.5°C, 31.0 to 35.0°C, 31.5 to 34.5°C, 32.0 to 34.0°C, or 32.5 to 33.5°C. Preferably, the cells are cultured at a decreased temperature of 30.0°C±1.0°C, 31.0°C±1.0°C, 32.0°C±1.0°C, 33.0°C±1.0°C, 34.0°C±1.0°C, or 35.0°C±1.0°C.

[0176] The decreased temperature is maintained for a time period that is sufficient to increase the sialylation of the polypeptide to be expressed. Preferably, the decreased temperature is maintained for at least 1 hour, at least 6 hours, at least 12 hours, at least 18 hours, at least 24 hours, at least 48 hours, at least 72 hours, at least 96 hours, at least 120 hours, or at least 144 hours. In other embodiments, the decreased temperature is maintained for 1 hour to 8 weeks, 6 hours to 6 weeks, 12 hours to 5 weeks, 18 hours to 4 weeks, 24 hours to 3 weeks, 48 hours to 14 days, 72 hours to 10 days, or 3 to 7 days.

[0177] To accomplish this, a culture may be subjected to one or more temperature shifts during the course of the culture. When shifting the temperature of a culture, the temperature shift may be relatively gradual. For example, it may take several hours or days to complete the temperature change. Alternatively, the temperature shift may be relatively abrupt. The temperature may be steadily increased or decreased during the culture process. Alternatively, the temperature may be increased or decreased by discrete amounts at various times during the culture process. The subsequent temperature(s) or temperature range(s) may be lower than or higher than the initial or previous temperature(s) or temperature range(s). One of ordinary skill in the art will understand that multiple discrete temperature shifts are encompassed in this embodiment. For example, the temperature may be shifted once (either to a higher or lower temperature or temperature range), the cells maintained at this temperature or temperature range for a certain period of time, after which the temperature may be shifted again to a new temperature or temperature range, which may be either higher or lower than the temperature or temperature range of the previous temperature or temperature range. The temperature of the culture after each discrete shift may be constant or may be maintained within a certain range of temperatures.

[0178] Typically, the cells (e.g. CHO cells) will initially be cultured at a "normal" temperature of $37.0^{\circ}\text{C} \pm 1.0^{\circ}\text{C}$ until the target cell density is achieved. The initial culture period is then followed by a temperature shift to the decreased temperature. After a period of culturing at the decreased temperature, a temperature shift to the normal temperature may or may not follow. Preferably, the cells (e.g. CHO cells) will initially be cultured at $37.0^{\circ}\text{C} \pm 1.0^{\circ}\text{C}$ for several days, followed by manufacturing at a decreased temperature of $31.0 - 35.0^{\circ}\text{C}$.

[0179] Based on the present disclosure, those of ordinary skill in the art will be able to select temperatures in which to grow cells, depending on the particular needs of the respective cell line and the particular production requirements of the practitioner.

[0180] In certain embodiments, batch and fed-batch bioreactors are terminated once the expressed polypeptide reaches a sufficiently high titer. Additionally or alternatively, batch and fed-batch bioreactors may be terminated once the cells reach a sufficiently high density, as determined by the needs of the practitioner. For example, the culture may be terminated once the cells reach 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 99 percent of maximal viable cell density. Additionally or alternatively, batch and fed-batch bioreactors may be terminated prior to excessive accumulation of metabolic waste products such as lactate and ammonium.

[0181] In certain cases, it may be beneficial to supplement a cell culture during the subsequent production phase with nutrients or other medium components that have been depleted or metabolized by the cells. As non-limiting examples, it may be beneficial to supplement a cell culture with hormones and/or other growth factors, inorganic ions (such as, for example, sodium, chloride, calcium, magnesium, and phosphate), buffers, vitamins, nucleosides or nucleotides, trace elements (inorganic compounds usually present at very low final concentrations), amino acids, lipids, or glucose or other energy source. Such supplementary

components may all be added to the cell culture at one time, or they may be provided to the cell culture in a series of additions or they may be provided together with fresh medium during a perfusion culture.

[0182] Alternatively to batch and fed-batch bioreactors the invention can also be practiced when cells expressing a polypeptide of the invention are cultured in continuous perfusion bioreactors.

[0183] One of ordinary skill in the art will be able to tailor specific cell culture conditions in order to optimize certain characteristics of the cell culture including but not limited to growth rate, cell viability, final cell density of the cell culture, final concentration of detrimental metabolic byproducts such as lactate and ammonium, titer of the expressed polypeptide, extent and composition of the oligosaccharide side chains or any combination of these or other conditions deemed important by the practitioner.

Isolation of the Expressed Polypeptide

[0184] In general, it will typically be desirable to isolate and/or purify polypeptides expressed according to the present invention. In certain embodiments, the expressed polypeptide is secreted into the medium and thus cells and other solids may be removed, as by centrifugation or filtering for example, as a first step in the purification process.

[0185] The expressed polypeptide may be isolated and purified by standard methods including, but not limited to, chromatography (e.g., ion exchange, affinity, size exclusion, and hydroxyapatite chromatography), gel filtration, centrifugation, or differential solubility, ethanol precipitation and/or by any other available technique for the purification of proteins (See, e.g., Scopes, Protein Purification Principles and Practice 2nd Edition, Springer-Verlag, New York, 1987; Higgins, S. J. and Hames, B. D. (eds.), Protein Expression: A Practical Approach, Oxford Univ Press, 1999; and Deutscher, M. P., Simon, M. I., Abelson, J. N. (eds.), Guide to Protein Purification: Methods in Enzymology (Methods in Enzymology Series, Vol. 182), Academic Press, 1997). For immunoaffinity chromatography in particular, the polypeptide may be isolated by binding it to an affinity column comprising antibodies that were raised against that polypeptide and were affixed to a stationary support. Alternatively, affinity tags such as an influenza coat sequence, poly-histidine, or glutathione-S-transferase can be attached to the polypeptide by standard recombinant techniques to allow for easy purification by passage over the appropriate affinity column. Protease inhibitors such as phenyl methyl sulfonyl fluoride (PMSF), leupeptin, pepstatin or aprotinin may be added at any or all stages in order to reduce or eliminate degradation of the polypeptide during the purification process. Protease inhibitors are particularly advantageous when cells must be lysed in order to isolate and purify the expressed polypeptide. Additionally or alternatively, glycosidase inhibitors may be added at any or all stages in order to reduce or eliminate enzymatic trimming of the covalently attached oligosaccharide chains.

[0186] Polypeptides expressed according to the present invention have more extensive sialylation than they would if grown under traditional cell culture conditions. Thus, one practical benefit of the present invention that may be exploited at the purification step is that the additional and/or altered sialic acid residues on a polypeptide grown in accordance with certain of the present inventive methods may confer on it distinct biochemical properties that may be used by the practitioner to purify that polypeptide more easily, or to a greater purity, than would be possible for a polypeptide grown in accordance with more traditional methods. For example, the polypeptide can be purified or greatly enriched by anion exchange chromatography, making use of the negative charge of the sialic acid residues. Thereby a further enrichment of polypeptide with high sialylation can be achieved.

[0187] In a further embodiment, the sialylation of the polypeptide obtained by a method of the invention can be further increased by contacting the polypeptide with a sialyltransferase in vitro. The sialyltransferase typically is a mammalian sialyltransferase, preferably it is a human sialyltransferase. The sialyltransferase may be an α -2,3-sialyltransferase and/or an α -2,6-sialyltransferase. Preferably, the sialyltransferase is a human α -2,3-sialyltransferase (Genbank NP_775479-ST3GAL 1) and/or a human α -2,6-sialyltransferase. Most preferably, the sialyltransferase is human α -2,6-sialyltransferase identified by Genbank NP_003023-ST6GAL 1). Further present in the in vitro reaction is a sialyl group donor, or sialic acid donor. Suitable donors include, e.g., Cytidine-5'-monophospho-N-acetylneuraminic acid (CMP-NANA), Roche Catalog No. 05 974 003 103. A suitable kit for in vitro sialylation is available from Roche (Catalog Number 07 012 250 103).

[0188] One of ordinary skill in the art will appreciate that the exact purification technique will vary depending on the character of the polypeptide to be purified, the character of the cells from which the polypeptide is expressed, and/or the composition of the medium in which the cells were grown.

[0189] As mentioned above, the invention, in a second aspect, relates to a method of producing a polypeptide comprising N-glycans with increased sialylation, which comprises (i) providing cells comprising a nucleic acid encoding a polypeptide comprising a truncated von Willebrand Factor (VWF) and a recombinant nucleic acid encoding an α -2,3-sialyltransferase and/or an α -2,6-sialyltransferase, preferably an α -2,6-sialyltransferase, and (ii) culturing the cells under conditions that allow expression of the polypeptide.

[0190] The α -2,3-sialyltransferase preferably is a human α -2,3-sialyltransferase. The cDNA sequence encoding human α -2,3-sialyltransferase is shown in SEQ ID NO:12, and based thereon the skilled artisan can design suitable expression vectors containing a coding sequence of α -2,3-sialyltransferase.

[0191] The α -2,6-sialyltransferase preferably is a human α -2,6-sialyltransferase. The cDNA sequence encoding human α -2,6-sialyltransferase is shown in SEQ ID NO:31, and based thereon the skilled artisan can design suitable expression vectors containing a coding sequence of α -2,6-sialyltransferase.

[0192] The transfected cells can be cultured under conditions allowing expression of the polypeptide according to known culturing methods.

[0193] The polypeptide can be recovered and/or isolated using established purification techniques.

Polypeptide of the invention

[0194] According to a preferred embodiment, the polypeptide of the invention comprises N-glycans, and at least 85%, more preferably at least 90% of said N-glycans comprise, on average, at least one sialic acid moiety. In preferred embodiments, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, of said N-glycans comprise, on average, at least one sialic acid moiety. The inventors demonstrated that polypeptides comprising highly sialylated VWF fragments not only have a prolonged half-life themselves, but are also capable to extend the half-life of co-administered FVIII. In other words, administration of the polypeptide of the invention leads to an extended half-life and/or to a reduced clearance of co-administered FVIII.

[0195] According to another preferred embodiment, the polypeptide of the invention comprises N-glycans, wherein at least 50% of the sialyl groups of the N-glycans of the polypeptides are α -2,6-linked sialyl groups. In general, terminal sialyl groups can be attached to the galactose groups via a α -2,3- or via a α -2,6-linkage. Typically, N-glycans of the polypeptide of the invention comprise more α -2,6-linked sialyl groups than α -2,3-linked sialyl groups. Preferably, at least 60%, or at least 70%, or at least 80%, or at least 90% of the sialyl groups of the N-glycans are α -2,6-linked sialyl groups. These embodiments can be obtained by, e.g., co-expressing human α -2,6-sialyltransferase in mammalian cells.

[0196] In one embodiment, at least 85%, at least 90%, or at least 95%, or at least 96%, or at least 97%, or at least 98%, or at least 99% of the N-glycans of the polypeptide of the invention comprise at least one sialic acid group. In another embodiment, at least 90%, or at least 95%, or at least 96%, or at least 97%, or at least 98%, or at least 99% of the N-glycans of the truncated VWF within the polypeptide of the invention comprise at least one sialic acid group.

[0197] In another embodiment, less than 15%, less than 12%, less than 10%, or less than 8%, or less than 6%, or less than 5%, or less than 4%, or less than 3%, or less than 2% or even less than 1% of the N-glycans of the polypeptide of the invention are asialo-N-glycans, i.e. they are N-glycans lacking a sialic acid group. In another embodiment, less than 15%, less than 12%, less than 10%, or less than 8%, or less than 6%, or less than 5%, or less than 4%, or less than 3%, or less than 2% or even less than 1% of the N-glycans of the truncated VWF within the polypeptide of the invention are asialo-N-glycans, i.e. they do not have a sialic acid group.

[0198] In another embodiment, less than 25%, less than 20%, less than 15%, or less than

10%, of the N-glycans of the polypeptide of the invention are monosialo-N-glycans, i.e. they are N-glycans with one sialic acid group. In another embodiment, less than 25%, less than 20%, less than 15%, or less than 10%, of the N-glycans of the truncated VWF within the polypeptide of the invention are monosialo-N-glycans, i.e. they are N-glycans with one sialic acid group. By way of non-limiting example the amount of monosialylated N-glycans can be determined as detailed in Example 6.

[0199] In yet another embodiment, at least 20%, or at least 25%, of the N-glycans of the polypeptide of the invention are disialo-N-glycans, i.e. they are N-glycans with 2 sialic acid groups. In yet another embodiment, at least 20%, or at least 25%, of the N-glycans of the truncated VWF within the polypeptide of the invention are disialo-N-glycans.

[0200] In yet another embodiment, at least 10%, or at least 15%, or at least 20%, or at least 25%, of the N-glycans of the polypeptide of the invention are trisialo-N-glycans, i.e. they are N-glycans with 3 sialic acid groups. In yet another embodiment, at least 10%, or at least 15%, or at least 20%, or at least 25%, of the N-glycans of the truncated VWF within the polypeptide of the invention are trisialo-N-glycans.

[0201] In yet another embodiment, at least 5%, or at least 10%, of the N-glycans of the polypeptide of the invention are tetrasialo-N-glycans, i.e. they are N-glycans with 4 sialic acid groups. In yet another embodiment, at least 10%, or at least 15%, of the N-glycans of the truncated VWF within the polypeptide of the invention are tetrasialo-N-glycans.

[0202] In another embodiment, at least 50%, or at least 60%, or at least 70%, or at least 80%, of the N-glycans of the polypeptide of the invention comprise two or more sialic acid groups. In another embodiment, at least 50%, or at least 60%, or at least 70%, or at least 80%, of the N-glycans of the truncated VWF within the polypeptide of the invention comprise two or more sialic acid groups.

[0203] The above-described embodiments can be combined with each other. Any percentages of N-glycans mentioned above, or any indications of the degree of sialylation, are to be understood as average percentages or degrees, i.e. they refer to a population of molecules, not to a single molecule. It is clear that the glycosylation or sialylation of the individual polypeptide molecules within a population of polypeptides will show some heterogeneity.

[0204] Another aspect of the present invention is a pharmaceutical kit comprising (i) a polypeptide as defined hereinabove and (ii) a Factor VIII. Preferably, the polypeptide and the FVIII are contained in separate compositions.

[0205] Another aspect of the present invention is a pharmaceutical kit comprising (i) a polypeptide as defined hereinabove and (ii) a Factor VIII, for simultaneous, separate or sequential use in the treatment of a blood coagulation disorder.

[0206] A summary of the sequences referred to herein is set out in Table 3.

Table 3

SEQ ID NO:	Description
1	Nucleotide sequence of DNA encoding SEQ ID NO:2
2	Amino acid sequence of human VWF pre-propolypeptide
3	Amino acid sequence of D'-D3 domains of human VWF
4	Amino acid sequence of mature human VWF
5	Truncated VWF including mutations S764P/S766W/V1083A
6	Truncated VWF including mutations S764G/S766Y/V1083A
7	Truncated VWF including mutations S764E/S766Y/V1083A
8	Truncated VWF including mutations N1011S/V1083A/K1181E
9	Truncated VWF including mutation V1083A
10	Truncated VWF including mutation S1042T
11	Truncated VWF including mutations V805A/Q1158L
12	Truncated VWF including mutations K912E/T1088S
13	Truncated VWF including mutation L781 P
14	Amino acid sequence of human Factor VIII
15	Amino acid sequence of a mature single-chain Factor VIII
16	Amino acid sequence of human serum albumin
17	Truncated VWF including mutations S766Y/V1083A
18	Truncated VWF including mutations S764G/S766Y
19	Truncated VWF including mutations S764P/S766I
20	Truncated VWF including mutations S764P/S766M
21	Truncated VWF including mutations S764V/S766Y
22	Truncated VWF including mutations S764E/S766Y
23	Truncated VWF including mutations S764Y/S766Y
24	Truncated VWF including mutations S764L/S766Y
25	Truncated VWF including mutations S764P/S766W
26	Truncated VWF including mutations S766W/S806A
27	Truncated VWF including mutations S766Y/P769K
28	Truncated VWF including mutations S766Y/P769N
29	Truncated VWF including mutations S766Y/P769R
30	Truncated VWF including mutations S764P/S766L
31	cDNA encoding human α -2,6-sialyltransferase

EXAMPLES

EXAMPLE 1***vWF mutants with improved FVIII binding******Background***

[0207] As discussed above and in co-pending International Patent Application No. PCT/AU2015/050369 the majority of circulating FVIII is in complex with VWF. In humans, FVIII is cleared from the blood with a $t_{1/2}$ of approximately 2hr and 16hr in the absence and presence of VWF, respectively. Although VWF imparts an increase in FVIII half-life, it also places an upper limit on the $t_{1/2}$ that is dictated by its own half-life. US 8,575,104 discloses a VWF-albumin fusion protein. This fusion protein has a five-fold longer half-life than wild type VWF in a rodent model. A stable complex between this fusion protein and FVIII may confer additional half-life benefits for FVIII. Although the equilibrium binding constant for the FVIII/vWF interaction is high, the binding kinetics are rapid and any FVIII in complex with the VWF-albumin fusion protein will quickly exchange with endogenous vWF upon infusion. Accordingly if the off-rate of FVIII with VWF-albumin fusion is substantially equivalent to the off-rate of FVIII with native VWF then the use of the VWF-albumin fusion will not provide any substantial increase in the half life of FVIII.

[0208] Accordingly, in order to take advantage of the longer half life of the VWF-albumin fusion to extend the half life of FVIII it is necessary to decrease the off-rate of FVIII with the VWF-albumin fusion. From modeling studies taking advantage of measurement made in patients with Type 2N von Willebrand disease in which the level of VWF is normal but the ability of the VWF to associate with FVIII is severely diminished it has been estimated that at least a five fold decrease in off-rate is required to provide a clinically relevant improvement in FVIII half life. The postulated relationship between decrease in FVIII VWF-albumin fusion off-rate and increase in FVIII half life is set out in Table 4.

Table 4

Decrease in FVIII VWF-albumin fusion off-rate	Postulated increase in FVIII half life (For 50 IU/kg of FVIII and 100 IU/kg of VWF with the VWF 5x half life extended)
2 fold	2.2
3 fold	2.6
5 fold	3
10 fold	3.6
20 fold	4.1

[0209] In an effort to decrease FVIII VWF-albumin fusion off-rate experiments were conducted to assess whether mutant VWF-albumin fusion protein may provide a significantly slower FVIII off-rate thereby providing a viable option to extend the half-life of FVIII through stable association with the VWF-albumin fusion protein.

[0210] A series of mutants were constructed around amino acid positions 764, 765, 766, 768, 769, 773, 806 and 809 of vWF with the intention of slowing the rate of dissociation of bound FVIII. In these experiments a recombinant form of FVIII was used. This FVIII is described in Zollner et al 2013, Thrombosis Research, 132:280-287. Initially, FVIII binding was measured for vWF constructs that had one of the above mentioned residues mutated to all genetic encoded amino acids, excluding cysteine. Following identification of improved binders additional sets of variants were produced including combinations of mutations. In addition, as the half life extension provided by the albumin fusion is dependent on FcRn-mediated recycling a number of the mutants were also tested at a pH 5.5. The results for the various mutations are shown in Tables 5 to 20.

Methods

[0211] A synthetic, codon-optimised cDNA encoding the D' and D3 domains of human von Willebrand Factor (vWF; amino acids (aa) 764-1270 (SEQ ID NO:2); based on GenBank accession no. NP_000543 was obtained from GeneART AG (Regensburg, Germany). This was modified at the 5' end to encode its own signal peptide (aa1-22) and at the 3' end to encode a C-terminal 8xHis-tag. The construct (Hu-vWF[764-1270]-8His) was directionally cloned into the pcDNA3.1 mammalian expression vector (Invitrogen, USA) with a Kozak consensus sequence (GCCACC) upstream of the initiating methionine and a double stop codon (TGA) at the 3' end of the open reading frame, and the plasmid sequence confirmed by automated sequencing. This expression plasmid was then used as a template to make single, double or triple residue changes at Ser764, Leu765, Ser766 or Lys773 using standard PCR techniques and the constructs cloned into pcDNA3.1 and sequenced as described above. A second codon-optimised cDNA encoding the D1 and D2 domains (aa1-762) of Hu-vWF with a C-terminal FLAG tag (DYKDDDDK) was also synthesized and obtained from GeneArt; this was cloned as above into pcDNA3.1 and sequenced.

[0212] For transient mammalian expression, Freestyle™ 293 suspension cells (Invitrogen) were grown to 1.1×10^6 cells/ml in 5ml Freestyle Expression media (Invitrogen). 7 µL 293Fectin (Invitrogen) transfection reagent was pre-incubated for 5 minutes with 167 µL Opti-MEM I medium (Invitrogen), then added to 2.5 µg plasmid DNA encoding wild-type / mutant Hu-vWF[764-1270]-8His plus 2.5 µg plasmid DNA encoding Hu-vWF[1-762]-FLAG and the mixture incubated for a further 20 minutes. The DNA-293Fectin complex was added to the cells which were cultured for 6 days at 37 °C, 8% CO₂ in a shaking incubator at 250 rpm.

Culture supernatants were harvested by centrifugation at 2000 rpm for 5 minutes and stored at 4 °C for analysis.

[0213] Binding kinetics were investigated by surface plasmon resonance using a Biacore 4000 biosensor at 37°C. Each mutant was captured from cell culture medium to a density of 40-150RU on a CM-5 sensor chip pre-immobilised with anti-His antibody (14,000 RU). In an initial screening study, FVIII was injected over the captured mutants for 5 minutes at 1nM and dissociation monitored for 5 minutes. Mutants that showed a decrease in k_d relative to wild-type were then re-examined with FVIII injected for 5 minutes at 1, 0.5 and 0.25nM, and dissociation monitored for 30 minutes.

[0214] All sensorgrams were double referenced by subtraction of signals from a reference spot (containing only immobilised anti His antibody) and from a blank injection. Binding kinetics were determined by fitting the double referenced sensorgrams to a 1:1 kinetic model.

Results

[0215] Mutagenesis of serine 764 to proline generated a vWF variant with an approximately 3.5 fold decrease in off-rate and a 4.4 fold increase in affinity. Mutations at position 765 did not yield any better binders vis-a-vis wild type vWF. Numerous mutations at position 766 generated variant vWF molecules with improved off-rate characteristics and higher affinity than wild-type vWF (His, Arg, Val, Tyr, Trp, Thr, Phe, Ile, Gln, Gly & Asn). Given that proline at position 764 conferred significant enhancement to off-rate while numerous mutations at position 766 positively impacted binding, a series of mutants were generated that consisted of S764P and all other genetic encoded amino acids, excluding cysteine, at position 766. Similar mutations were produced that contained S764P and all other genetic encoded amino acids, excluding cysteine, at position 765. A number of these double mutants have significantly slower off-rates and higher affinity vis-a-vis wild type vWF. In particular S764P in combination with S766I generates a vWF variant with a 22 fold decrease in off-rate and a 30 fold increase in affinity.

EXAMPLE 2

Human serum albumin vWF fusions with point mutants and FVIII binding

[0216] Subsequent experiments were conducted using vWF fused to human serum albumin. A synthetic, codon-optimised cDNA encoding the D' and D3 domains of human von Willebrand Factor (vWF; amino acids (aa) 764-1242; based on GenBank accession no. NP_000543) was obtained from GeneART AG (Regensburg, Germany). This was modified at the 5' end to encode its own signal peptide (aa1-22) and at the 3' end to encode human serum albumin (HSA) via a glycine serine linker and cloned as described in Example 1. The same process as

described in Example 1 was used to generate the various VWF mutations and the resulting constructs were transiently transfected into Freestyle™ 293 suspension cells. vWF-HSA proteins were purified from harvests using Capture Select™ Human Albumin affinity resin and the vWF-HSA dimer further purified by preparative Size Exclusion Chromatography. Detailed kinetic analysis at pH7 was set up for the top candidates, including controls.

[0217] Mouse anti-HSA antibody was immobilized on a CM5 chip using standard NHS/EDC coupling chemistry. Typically, the immobilization level was between 10,000 and 12,000 RU. Each batch of vWF-HSA (monomers and dimers) was captured on a single spot in each flow cell for 2 minutes at various concentrations ranging from 0.1 - 1µg/ml. Capture levels ranged from 40-150RU. An adjacent spot in which anti-vWF was immobilized, but no vWF-HSA captured was used as a reference. Capture was performed every cycle, before FVIII binding analysis.

[0218] FVIII was injected at random and in duplicate over all spots in all flow cells at varying concentrations depending on the affinity of the interaction and the pH of the analysis. The association and dissociation of FVIII was monitored for various time frames that best suited the interaction taking place.

[0219] Post the dissociation period the surface was regenerated with a 30 second injection of 25mM Glycine pH2.6. Running buffer throughout was 10mM HEPES, 150mM NaCl, 10mM Na Citrate, 2.5mM CaCl₂, 0.1%BSA, pH7.3 and pH5, while the flow rate was 30 µl/min. Each interaction was measured 4 times (n=4) at 37°C.

[0220] Responses for binding to the reference spot were subtracted from those of the vWF-HSA captured spots. Responses from blank injections were then subtracted from those of all other samples to produce double-referenced sensorgrams. Double referenced sensorgrams were fitted to a 1:1 kinetic model, including a term for mass transport limitation. Association and dissociation rates were fitted globally and Rmax fitted locally. The results obtained are set out in Tables 21 and 22.

Table 5

S764X mutants where X is one of the remaining genetic encoded amino acids, excluding cysteine.			
Mutant	ka (1/Ms)	kd (1/s)	KD (M)
S764P	9.07E+06	3.25E-04	3.58E-11
S764Y	8.07E+06	8.87E-04	1.10E-10
S764E	6.38E+06	7.43E-04	1.16E-10
S764L	8.47E+06	9.95E-04	1.18E-10
S764A	6.85E+06	8.08E-04	1.18E-10
S764G	6.82E+06	8.18E-04	1.20E-10
S764I	9.02E+06	1.27E-03	1.41E-10

S764X mutants where X is one of the remaining genetic encoded amino acids, excluding cysteine.

Mutant	ka (1/Ms)	kd (1/s)	KD (M)
S764W	9.46E+06	1.41E-03	1.49E-10
wt	7.33E+06	1.15E-03	1.57E-10
wt	7.43E+06	1.18E-03	1.59E-10
S76R	1.06E+07	1.77E-03	1.67E-10
S764F	8.14E+06	1.40E-03	1.72E-10
S764N	6.21E+06	1.26E-03	2.03E-10
S764M	8.94E+06	1.90E-03	2.12E-10
S764V	7.30E+06	1.69E-03	2.32E-10
S764T	7.17E+06	1.89E-03	2.64E-10
S764D	6.27E+06	1.68E-03	2.68E-10
S76H	8.96E+06	2.78E-03	3.10E-10
S76K	1.59E+07	5.09E-03	3.19E-10
S764Q	2.97E+06	2.04E-03	6.86E-10

Table 6

L765X mutants where X is one of the remaining genetic encoded amino acids, excluding cysteine.

Mutant	ka (1/Ms)	kd (1/s)	KD (M)
WT-L765A	3.40E+07	7.88E-03	2.32E-10
WT-L765N	N/D		
WT-L765Q	N/D		
WT-L765G	N/D		
WT-L765I	6.01E+06	1.16E-03	1.92E-10
WT-L765M	6.81E+06	1.95E-03	2.87E-10
WT-L765F	8.91E+06	1.74E-03	1.96E-10
WT-L765P	1.13E+08	4.80E-02	4.25E-10
WT-L765S	3.46E+07	9.13E-03	2.64E-10
WT-L765T	7.53E+07	1.75E-02	2.32E-10
WT-L765W	3.53E+07	1.42E-02	4.03E-10
WT-L765Y	8.44E+07	4.36E-02	5.17E-10
WT-L765V	6.24E+06	4.76E-03	7.63E-10
WT-L765D	N/D		
WT-L765E	N/D		
WT-L765R	1.32E+08	1.55E-02	1.17E-10

L765X mutants where X is one of the remaining genetic encoded amino acids, excluding cysteine.

Mutant	ka (1/Ms)	kd (1/s)	KD (M)
WT-L765H	N/D		
WT-L765K	N/D		
WT	7.33E+06	1.15E-03	1.57E-10

N/D : weak binding, poor fit, fast off rate

Table 7

S766X mutants where X is one of the remaining genetic encoded amino acids, excluding cysteine.

Mutant	ka (1/Ms)	kd (1/s)	KD (M)
WT-S766A	7.47E+06	1.54E-03	2.06E-10
WT-S766N	8.71E+06	8.80E-04	1.01E-10
WT-S766Q	7.42E+06	5.16E-04	6.94E-11
WT-S766G	9.34E+06	1.88E-03	2.01E-10
WT-S766I	6.17E+06	7.93E-04	1.29E-10
WT-S766L	7.31E+06	1.21E-03	1.65E-10
WT-S766M	N/D		
WT-S766F	7.46E+06	2.74E-04	3.67E-11
WT-S766P	1.16E+07	3.45E-03	2.98E-10
WT-S766T	7.12E+06	4.98E-04	7.00E-11
WT-S766W	6.62E+06	2.03E-04	3.07E-11
WT-S766Y	6.98E+06	1.95E-04	2.79E-11
WT-S766V	6.01E+06	2.60E-04	4.33E-11
WT-S766D	N/D		
WT-S766E	2.53E+07	1.89E-03	7.48E-11
WT-S766R	9.04E+06	3.63E-04	4.02E-11
WT-S766H	7.19E+06	3.06E-04	4.25E-11
WT-S766K	1.02E+07	3.22E-03	3.14E-10
WT	7.33E+06	1.15E-03	1.57E-10

N/D : weak binding, poor fit, fast off-rate

Table 8

Mutant	Ka (1/Ms)	kd (1/s)	KD (M)
WT-K773T	1.42E+07	6.97E-04	4.92E-11
WT-K773A	5.81E+06	8.83E-04	1.52E-10
WT-K773L	1.88E+07	1.10E-03	5.86E-11

Mutant	Ka (1/Ms)	kd (1/s)	KD (M)
WT-K773R	1.45E+07	1.23E-03	8.46E-11
WT-K773Q	8.60E+06	1.45E-03	1.68E-10
WT-K773M	1.57E+07	2.35E-03	1.50E-10
WT-K773S	1.35E+07	3.23E-03	2.40E-10
WT-K773P	9.58E+06	3.33E-03	3.48E-10
WT-K773I	7.66E+07	4.09E-03	5.35E-11
WT-K773V	5.39E+07	5.23E-03	9.70E-11
WT-K773H	1.19E+09	1.57E-01	1.32E-10
WT-K773N	3.61E+09	8.36E-01	2.32E-10
WT-K773W	N/D		
WT-K773E	N/D		
WT-K773D	N/D		
WT-K773G	N/D		
WT-K773F	N/D		
WT-K773Y	N/D		
WT	7.33E+06	1.15E-03	1.57E-10

N/D: Binding was present, but accurate kinetic parameters could not be determined

Table 9

S764P, L765X mutants where X is one of the remaining genetic encoded amino acids, excluding cysteine.

Mutant	ka (1/Ms)	kd (1/s)	KD (M)
S764P-L765A	3.07E+07	2.78E-02	9.06E-10
S764P-L765N	N/D		
S764P-L765Q	8.12E+06	7.14E-03	8.80E-10
S764P-L765G	N/D		
S764P-L765I	8.08E+06	9.52E-05	1.18E-11
S764P-L765M	9.76E+06	2.37E-04	2.43E-11
S764P-L765F	1.69E+07	6.32E-04	3.73E-11
S764P-L765P	1.02E+07	2.42E-04	2.38E-11
S764P-L765S	N/D		
S764P-L765T	1.39E+07	8.82E-03	6.34E-10
S764P-L765W	7.97E+06	5.14E-03	6.45E-10
S764P-L765Y	6.19E+06	2.20E-03	3.55E-10
S764P-L765V	6.19E+06	2.20E-03	3.55E-10
S764P-L765D	N/D		

S764P, L765X mutants where X is one of the remaining genetic encoded amino acids, excluding cysteine.

Mutant	k_a (1/Ms)	k_d (1/s)	KD (M)
S764P-L765E	N/D		
S764P-L765R	N/D		
S764P-L765H	1.16E+07	6.42E-03	5.55E-10
S764P-L765K	N/D		
WT	7.33E+06	1.15E-03	1.57E-10

N/D : weak binding, poor fit, fast off-rate

Table 10

S764P, S766X mutants where X is one of the remaining genetic encoded amino acids, excluding cysteine.

Mutant	k_a (1/Ms)	k_d (1/s)	KD (M)
S764P-S766A	1.35E+07	1.66E-04	1.23E-11
S764P-S766N	8.82E+06	9.14E-05	1.04E-11
S764P-S766Q	1.20E+07	1.23E-04	1.02E-11
S764P-S766G	1.79E+07	3.88E-04	2.17E-11
S764P-S766I	9.84E+06	5.14E-05	5.23E-12
S764P-S766L	1.44E+07	8.74E-05	6.06E-12
S764P-S766M	1.18E+07	5.76E-05	4.88E-12
S764P-S766F	1.35E+07	1.00E-04	7.41E-12
S764P-S766P	2.56E+07	2.17E-03	8.48E-11
S764P-S766T	9.01E+06	1.05E-04	1.16E-11
S764P-S766W	1.10E+07	8.00E-05	7.27E-12
S764P-S766Y	1.08E+07	7.71E-05	7.16E-12
S764P-S766V	8.19E+05	7.82E-05	9.56E-11
S764P-S766D	9.41E+06	1.20E-04	1.27E-11
S764P-S766E	8.04E+06	1.28E-04	1.60E-11
S764P-S766R	1.29E+07	1.19E-04	9.21E-12
S764P-S766H	1.40E+07	9.47E-05	6.76E-12
S764P-S766K	2.15E+07	3.01E-04	1.40E-11
WT	7.33E+06	1.15E-03	1.57E-10

N/D : weak binding, poor fit, fast off-rate

Table 11

Mutant	k_a (1/Ms)	k_d (1/s)	KD (M)
S764P-K773R	6.39E+06	7.42E-05	1.16E-11

Mutant	ka (1/Ms)	kd (1/s)	KD (M)
S764P-K773T	4.68E+06	7.50E-05	1.60E-11
S764P-K773Q	4.44E+06	1.28E-04	2.88E-11
S764P-K773V	1.55E+07	1.57E-04	1.01E-11
S764P-K773I	1.79E+07	1.69E-04	9.43E-12
S764P-K773M	1.58E+07	1.70E-04	1.08E-11
S764P-K773A	6.37E+06	1.89E-04	2.97E-11
S764P-K773S	2.16E+07	3.06E-04	1.42E-11
S764P-K773N	5.50E+06	3.47E-04	6.31E-11
S764P-K773P	2.26E+07	5.01E-04	2.22E-11
S764P-K773L	4.60E+05	5.72E-04	1.24E-09
S764P-K773H	1.65E+07	6.36E-04	3.86E-11
S764P-K773G	1.75E+07	7.62E-04	4.36E-11
S764P-K773F	1.02E+07	1.23E-03	1.21E-10
S764P-K773Y	1.63E+07	1.36E-03	8.35E-11
S764P-K773D	1.77E+07	2.40E-03	1.36E-10
S764P-K773W	1.25E+07	3.21E-03	2.57E-10
S764P-K773E	6.73E+07	5.15E-03	7.65E-11
WT	7.33E+06	1.15E-03	1.57E-10

Table 12

Mutant	ka (1/Ms)	kd (1/s)	KD (M)
S766Y-K773T	1.20E+07	2.69E-04	2.24E-11
S766Y-K773L	1.79E+07	3.45E-04	1.92E-11
S766Y-K773R	1.40E+07	4.69E-04	3.35E-11
S766Y-K773I	8.02E+06	5.69E-04	7.10E-11
S766Y-K773M	1.97E+07	6.59E-04	3.35E-11
S766Y-K773V	1.74E+07	8.61E-04	4.94E-11
S766Y-K773Q	2.39E+07	9.39E-04	3.93E-11
S766Y-K773A	1.88E+07	1.22E-03	6.51E-11
S766Y-K773S	1.75E+07	1.38E-03	7.85E-11
S766Y-K773G	6.02E+07	1.97E-03	3.27E-11
S766Y-K773P	2.16E+07	2.43E-03	1.12E-10
S766Y-K773F	2.05E+07	3.24E-03	1.58E-10
S766Y-K773W	2.93E+07	3.93E-03	1.34E-10
S766Y-K773Y	2.24E+07	4.04E-03	1.80E-10
S766Y-K773E	1.84E+07	4.81E-03	2.61E-10

Mutant	ka (1/Ms)	kd (1/s)	KD (M)
S766Y-K773N	5.15E+07	5.07E-03	9.84E-11
S766Y-K773H	5.47E+07	6.20E-03	1.14E-10
S766Y-K773D	1.25E+08	4.27E-02	3.43E-10
WT	7.33E+06	1.15E-03	1.57E-10

Table 13

Mutant	ka (1/Ms)	kd (1/s)	KD (M)
S764G/S766Y	1.37E+07	2.69E-05	1.96E-12
S764V/S766Y	2.99E+07	6.41E-05	2.15E-12
S764A/S766Y	2.98E+07	7.21E-05	2.42E-12
S764E/S766Y	1.97E+07	7.64E-05	3.87E-12
S764P-S766Y	1.08E+07	7.71E-05	7.16E-12
S764Y/S766Y	3.19E+07	7.88E-05	2.47E-12
S764L/S766Y	3.52E+07	7.99E-05	2.27E-12
S764N/S766Y	1.28E+07	8.88E-05	6.92E-12
S764R/S766Y	3.23E+07	9.20E-05	2.85E-12
S764F/S766Y	7.68E+06	9.36E-05	1.22E-11
S764I/S766Y	1.03E+07	9.52E-05	9.23E-12
S764W/S766Y	8.88E+06	9.67E-05	1.09E-11
S764M/S766Y	7.15E+06	1.03E-04	1.44E-11
S764Q/S766Y	1.19E+07	1.09E-04	9.18E-12
S764D/S766Y	3.78E+07	1.18E-04	3.12E-12
S764T/S766Y	2.58E+07	1.36E-04	5.27E-12
S764H/S766Y	4.56E+07	2.92E-04	6.39E-12
S764K/S766Y	1.89E+07	8.22E-04	4.35E-11
WT	7.33E+06	1.15E-03	1.57E-10

Table 14

Mutant	ka (1/Ms)	kd (1/s)	KD (M)
S764P-L765H-S766I	1.56E+06	6.60E-05	4.24E-11
S764P-L765V-S766I	5.62E+07	1.16E-04	2.07E-12
S764P-L765M-S766I	5.69E+07	1.37E-04	2.41E-12
S764P-L765W-S766I	1.11E+06	1.46E-04	1.32E-10
S764P-L765Q-S766I	1.15E+06	2.86E-04	2.48E-10
S764P-L765K-S766I	6.88E+07	1.50E-03	2.18E-11
S764P-L765Y-S766I	5.17E+07	1.90E-03	3.67E-11
S764P-L765T-S766I	1.15E+08	3.31E-03	2.87E-11

Mutant	ka (1/Ms)	kd (1/s)	KD (M)
S764P-L765I-S766I	6.34E+06	1.03E-02	1.62E-09
S764P-L765G-S766I	5.04E+07	1.22E-02	2.41E-10
S764P-L765R-S766I	7.96E+07	1.73E-02	2.18E-10
S764P-L765E-S766I	1.03E+06	5.50E-02	5.36E-08
S764P-L765F-S766I	N/D		
S764P-L765N-S766I	N/D		
S764P-L765D-S766I	N/D		
S764P-L765P-S766I	N/D		
S764P-L765S-S766I	N/D		
S764P-L765A-S766I	N/D		
N/D: Binding was present, but accurate kinetic parameters could not be determined			

Table 15

Mutant	ka (1/Ms)	kd (1/s)	KD (M)
dupS764/S764P/S766I	6.23E+06	1.59E-03	2.55E-10
dupS764/S764P/S766I	1.25E+07	2.50E-03	1.99E-10
dS764-dL765-S766I			
dS764-dL765-S766Y	N/D		
delS764-S766Y	6.20E+06	2.07E-04	3.34E-11
delS764-S766W	6.60E+06	3.15E-04	4.78E-11
delS764-S766L	6.21E+06	5.85E-04	9.42E-11
delS764-S766M	7.25E+06	7.26E-04	1.00E-10
delS764-S766I	7.09E+06	8.27E-04	1.17E-10
delS764-S766S	7.30E+06	8.46E-04	1.16E-10
N/D: Binding was present, but accurate kinetic parameters could not be determined			

Table 16

PH 5.5			
Mutant	ka (1/Ms)	kd (1/s)	KD (M)
S764P-S766W	2.77E+05	4.75E-05	1.72E-10
S764P-S766M	3.14E+05	9.16E-05	2.92E-10
S764P-S766L	4.45E+05	1.04E-04	2.34E-10
WT	2.03E+06	3.88E-02	1.91E-08
S764P-S766I	N/D		
S764P-S766Y	N/D		
S764P-S766H	N/D		

N/D: Binding was present, but accurate kinetic parameters could not be determined

Table 17

S766W, L809X mutants where X is one of the remaining genetic encoded amino acids, excluding cysteine

Mutant	ka (1/Ms)	kd (1/s)	KD (M)
S766W-L809A	4.45E+06	1.15E-03	2.58E-10
S766W-L809D	4.46E+06	1.90E-03	4.25E-10
S766W-L809E	5.84E+06	1.55E-03	2.65E-10
S766W-L809F	3.26E+06	7.44E-04	2.28E-10
S766W-L809G	6.21E+06	2.26E-03	3.63E-10
S766W-L809H	2.87E+06	1.14E-03	3.97E-10
S766W-L809I	5.23E+06	5.41E-04	1.03E-10
S766W-L809K	7.00E+06	1.53E-03	2.19E-10
S766W-L809M	4.99E+06	5.81E-04	1.17E-10
S766W-L809N	6.15E+06	2.27E-03	3.69E-10
S766W-L809P	NB	NB	NB
S766W-L809Q	5.33E+06	1.13E-03	2.12E-10
S766W-L809R	6.07E+06	2.13E-03	3.52E-10
S766W-L809S	6.54E+06	1.44E-03	2.20E-10
S766W-L809T	8.72E+06	1.41E-03	1.61E-10
S766W-L809V	7.70E+06	9.40E-04	1.22E-10
S766W-L809W	4.81E+06	3.12E-03	6.48E-10
S766W-L809Y	6.77E+06	3.39E-03	5.00E-10
vWF WT	4.98E+06	8.86E-04	1.78E-10

Table 18

S766W, S806X mutants where X is one of the remaining genetic encoded amino acids, excluding cysteine

Mutant	ka (1/Ms)	kd (1/s)	KD (M)
S766W-S806A	4.84E+06	3.76E-04	7.78E-11
S766W-S806D	4.20E+06	6.88E-04	1.64E-10
S766W-S806E	5.93E+06	1.29E-03	2.17E-10
S766W-S806F	NB	NB	NB
S766W-S806G	5.46E+06	1.34E-03	2.45E-10
S766W-S806H	8.90E+06	8.28E-04	9.30E-11
S766W-S806I	1.58E+06	4.47E-04	2.83E-10
S766W-S806K	N/D		

S766W, S806X mutants where X is one of the remaining genetic encoded amino acids, excluding cysteine

Mutant	ka (1/Ms)	kd (1/s)	KD (M)
S766W-S806L	NB	NB	NB
S766W-S806M	2.05E+06	8.72E-04	4.25E-10
S766W-S806N	3.84E+06	5.85E-04	1.52E-10
S766W-S806P	4.26E+06	5.66E-04	1.33E-10
S766W-S806Q	4.33E+06	1.76E-03	4.07E-10
S766W-S806R	8.28E+06	1.07E-02	1.29E-09
S766W-S806T	5.25E+06	6.54E-04	1.25E-10
S766W-S806V	4.17E+06	6.19E-04	1.49E-10
S766W-S806W	NB	NB	NB
S766W-S806Y	NB	NB	NB
vWF WT	4.98E+06	8.86E-04	1.78E-10

N/D: Binding was present, but accurate kinetic parameters could not be determined

Table 19

S766Y, P769X mutants where X is one of the remaining genetic encoded amino acids, excluding cysteine

Mutant	ka (1/Ms)	kd (1/s)	KD (M)
S766Y-P769A	4.90E+06	5.19E-04	1.06E-10
S766Y-P769D	4.63E+06	7.63E-04	1.65E-10
S766Y-P769E	4.42E+06	4.14E-04	9.36E-11
S766Y-P769F	5.54E+06	4.27E-04	7.72E-11
S766Y-P769G	3.70E+06	7.83E-04	2.12E-10
S766Y-P769H	5.16E+06	4.17E-04	8.09E-11
S766Y-P769I	NB	NB	NB
S766Y-P769K	6.31E+06	3.83E-04	6.07E-11
S766Y-P769L	6.44E+06	5.90E-04	9.17E-11
S766Y-P769M	4.75E+06	5.11E-04	1.08E-10
S766Y-P769N	1.60E+07	5.20E-04	3.25E-11
S766Y-P769Q	NB	NB	NB
S766Y-P769R	6.55E+06	2.95E-04	4.50E-11
S766Y-P769S	4.51E+06	5.11E-04	1.13E-10
S766Y-P769T	5.11E+06	5.00E-04	9.79E-11
S766Y-P769V	6.65E+06	5.65E-04	8.49E-11
S766Y-P769W	4.77E+06	4.21E-04	8.82E-11

S766Y, P769X mutants were X is one of the remaining genetic encoded amino acids, excluding cysteine

Mutant	ka (1/Ms)	kd (1/s)	KD (M)
S766Y-P769Y	4.68E+06	3.96E-04	8.47E-11
vWF WT	4.98E+06	8.86E-04	1.78E-10

Table 20

S766Y, R768X mutants were X is one of the remaining genetic encoded amino acids, excluding cysteine

Mutant	ka (1/Ms)	kd (1/s)	KD (M)
S766Y-R768A	6.99E+06	1.48E-03	2.12E-10
S766Y-R768D	4.94E+06	4.48E-03	9.08E-10
S766Y-R768E	5.65E+06	3.22E-03	5.69E-10
S766Y-R768F	6.51E+06	1.82E-03	2.79E-10
S766Y-R768G	3.20E+06	1.02E-03	3.20E-10
S766Y-R768H	4.02E+06	6.90E-04	1.72E-10
S766Y-R768I	5.03E+06	8.99E-04	1.79E-10
S766Y-R768K	3.83E+06	4.17E-04	1.09E-10
S766Y-R768L	4.24E+06	5.48E-04	1.29E-10
S766Y-R768M	4.08E+06	8.01E-04	1.96E-10
S766Y-R768N	4.18E+06	7.98E-04	1.91E-10
S766Y-R768P	6.71E+06	1.43E-03	2.13E-10
S766Y-R768Q	3.48E+06	6.06E-04	1.74E-10
S766Y-R768S	5.33E+06	1.29E-03	2.43E-10
S766Y-R768T	5.59E+06	1.43E-03	2.56E-10
S766Y-R768V	4.51E+06	9.18E-04	2.03E-10
S766Y-R768W	4.42E+06	9.40E-04	2.13E-10
S766Y-R768Y	6.74E+06	1.87E-03	2.77E-10
vWF WT	4.98E+06	8.86E-04	1.78E-10

Table 21

Dimers Binding to FVIII (pH7.3)

Mutant	ka (1/Ms)	kd (1/s)	KD (M)
S764P-S766I	1.01E+07 ($\pm 3.41E6$)	5.00E-05 ($\pm 3.37E-6$)	3.96E-12 ($\pm 2.6E-13$)
S764P-S766W	1.24E+07 ($\pm 7.28E5$)	6.21E-05 ($\pm 2.52E-6$)	4.96E-12 ($\pm 1.9E-13$)
S766Y	1.03E+07 ($\pm 3.01E6$)	2.36E-04 ($\pm 4.27E-5$)	2.51E-11 ($\pm 3.83E-12$)
S764E-S766Y	7.75E+06 ($\pm 1.71E6$)	2.36E-04 ($\pm 2.90E-5$)	3.25E-11 ($\pm 4.57E-12$)
S764I-S766W	7.54E+06 ($\pm 5.15E5$)	2.41E-04 ($\pm 5.05E-6$)	3.25E-11 ($\pm 2.25E-12$)

Dimers Binding to FVIII (pH7.3)			
Mutant	ka (1/Ms)	kd (1/s)	KD (M)
S764G-S766Y	1.19E+07 (±9.1E5)	2.63E-04 (±1.41E-5)	2.29E-11 (±3.42E-12)
S766Y-P769R	1.18E+07 (±4.1E5)	2.75E-04 (±1.71E-5)	2.32E-11 (±9.54E-13)
S766Y-P769K	1.09E+07 (±1.37E6)	2.85E-04 (±2.08E-5)	2.68E-11 (±1.55E-12)
S766W-S806A	8.88E+06 (±1.11E6)	3.00E-04 (±1.9E-5)	3.54E-11 (±4.37E-12)
S764Y-S766Y	1.14E+07 (±1.71E6)	3.34E-04 (±2.7E-5)	3.07E-11 (±3.53E-12)
S766Y-S769N	1.21E+07 (±1.11E6)	3.48E-04 (±3.21E-5)	2.89E-11 (±1.75E-12)
S764A	1.26E+07 (±1.38E6)	6.38E-04 (±3.24E-5)	5.14E-11 (±2.81E-12)
WT	1.89E+07 (±2.68E6)	1.47E-03 (±8.92E-5)	8.25E-11 (±7.94E-12)

Table 22

Dimers Binding to FVIII (pH5.5)			
Mutant	ka (1/Ms)	kd (1/s)	KD (M)
S764P-S766I	3.10E+06 (±3.05E5)	1.81E-03 (±6.34E-5)	5.98E-10 (±4.93E-11)
S764P-S766W	3.02E+06 (±2.39E5)	1.88E-03 (±1.78E-5)	6.37E-10 (±5.75E-11)
S764E-S766Y	2.43E+06 (±1.6E5)	2.71E-03 (±9.8E-5)	1.12E-09 (±5.29E-11)
S764Y-S766Y	3.22E+06 (±1.24E5)	3.45E-03 (±9.01E-5)	1.07E-09 (±4.67E-11)
S766Y-P769R	4.66E+06 (±1.47E5)	6.54E-03 (±2.02E-4)	1.40E-09 (±2.29E-11)
S764I-S766W	3.28E+06 (±1.22E5)	7.24E-03 (±2.89E-4)	2.21E-09 (±5.78E-11)
S766Y-P769K	4.14E+06 (±2.95E5)	7.40E-03 (±3.9E-4)	1.79E-09 (±1.27E-10)
S766Y	3.50E+06 (±2.5E5)	7.40E-03 (±2.12E-3)	2.92E-09 (±1.38E-10)
S766Y-S769N	2.05E+06 (±2.02E5)	1.02E-02 (±7.84E-4)	5.01E-09 (±2.67E-10)
S766W-S806A	8.13E+05 (±2.83E5)	1.40E-02 (±6.74E-4)	1.43E-08 (±2.38E-9)
S764G-S766Y	2.66E+06 (±4.55E5)	1.85E-02 (±1.12E-3)	7.53E-09 (±1.15E-9)
S764A	2.25E+06 (±1.42E6)	4.01E-02 (±2.54E-3)	5.26E-08 (±3.33E-9)
WT	1.37E+06 (±2.44E5)	4.26E-02 (±3.9E-3)	3.54E-08 (±2.89E-9)

EXAMPLE 3

[0221] In an extension of the work described in PCT/AU2015/050369 further mutations and combinations of mutations were investigated with an emphasis on modifications in the D3 domain. In these experiments a recombinant form of FVIII was used. This FVIII is described in Zollner et al 2013, Thrombosis Research, 132:280-287.

Methods

[0222] Plasmid constructs encoding vWF(763-1242) -HSA and containing the single, double or triple mutations listed in Table 23 were used to generate purified vWF-HSA dimer proteins using the methods described in Example 2. Detailed kinetic analysis at pH7 was set up for the top candidates, including controls.

[0223] Mouse anti-HSA antibody was immobilized on a CM5 chip using standard NHS/EDC coupling chemistry. Typically, the immobilization level was about 14,000 RU. Each Dimer mutant of vWF-HSA was captured on a single spot in each flow cell for 2 minutes at various concentrations ranging from 0.1 - 1µg/ml. Capture levels ranged from 100-200RU. An adjacent spot in which anti-HSA was immobilized, but no vWF-HSA captured was used as a reference. Capture was performed every cycle, before FVIII binding analysis. In initial experiments, Factor VIII was injected at random and in duplicate over all spots and all flow cells in use at 5, 1 and 1.25nM. The results of this analysis are set out in Table 23.

Table 23: Screen at pH 7: affinities and kinetic rates of Factor VIII for various mutant D'D3-HSA dimer proteins ranked from strongest to weakest affinities.

Protein	ka(1/Ms)	kd(1/s)	KD (M)	Sample size
V1083A, S764P, S766W	6.15E+06	1.17E-04	1.90E-11	n=1
V1083A, S764G, S766Y	6.19E+06	1.86E-04	3.01E-11	n=2
N1011S,V1083A, K1181E	5.31E+06	4.30E-04	8.10E-11	n=1
V1083A	5.51E+06	5.39E-04	9.78E-11	n=1
S1042T	4.39E+06	4.69E-04	1.07E-10	n=2
V805A, Q1158L	4.26E+06	6.32E-04	1.49E-10	n=2
K912E, T1088S	5.17E+06	8.00E-04	1.55E-10	n=2
L781P	4.27E+06	6.99E-04	1.64E-10	n=2
WT	4.83E+06	1.08E-03	2.23E-10	n=1
R960G	4.09E+06	1.11E-03	2.72E-10	n=2
WT=wildtype				

EXAMPLE 4

Detailed Kinetic Analysis

[0224] Subsequent experiments were conducted where detailed kinetic analysis at pH7 was

set up for the top two candidates, including controls. Factor VIII was injected at 1, 0.5, 0.25, 0.125 and 0.06nM. In a similar manner detailed kinetic analysis on the top two candidates, including controls was set up at pH5.5 where Factor VIII was injected at various concentrations that best suited the interaction.

[0225] Throughout all experiments buffer blanks were also injected over all captured proteins. The association and dissociation of Factor VIII was monitored for 3 minutes respectively during the "screening" experiment. The association of CSL627 was monitored for 5 minutes and dissociation was monitored for 20 and 60 minutes during the "detailed kinetic analysis" experiments at neutral pH. At pH 5.5 the association and dissociation of Factor VIII was monitored for various time frames that best suited the interaction.

[0226] Post the dissociation period the surface was regenerated with a 45 second injection of 25mM Glycine pH2.6. Running buffer throughout was 10mM HEPES, 150mM NaCl, 10mM Na Citrate, 2.5mM CaCl₂, 0.1%BSA, pH7.3 and pH5.5, while the flow rate was 30 µl/min. Each interaction was measured at least 2 times (n=2) at 37°C.

[0227] Responses for binding to the reference spot were subtracted from those of the vWF-HSA captured spots. Responses from blank injections were then subtracted from those of all other samples to produce double-referenced sensorgrams. Double referenced sensorgrams were fitted to a 1:1 kinetic model, including a term for mass transport limitation. Association and dissociation rates were fitted globally and Rmax fitted locally. The results are set out in Tables 24 and 25 and shown in Figures 1-3.

Table 24: Detailed Kinetics at pH 7: affinities and kinetic rates of Factor VIII for mutant D'D3-HSA dimers.

Protein	ka (1/Ms)	kd (1/s)	KD (M)	Sample Size
V1083A,S764P, S766W (PWA)	1.15E+07	3.84E-05	3.36E-12	n=4
V1083A,S764G, S766Y (GYA)	1.63E+07	7.30E-05	4.50E-12	n=2
S764G, S766Y (GY)	2.95E+07	3.65E-04	1.25E-11	n=3
WT*	1.06E+07	8.46E-04	8.03E-11	n=2
WT=wildtype				

Table 25: Detailed Kinetics at pH5.5: affinities and kinetic rates of Factor VIII for mutant D'D3-HSA dimers

Protein	ka (1/Ms)	kd (1/s)	KD (M)	Sample Size
V1083A,S764P,S766W	2.51 E+06	8.44E-04	3.42E-10	n=4
S764P,S766W	2.94E+06	1.80E-03	6.14E-10	n=2
V1083A,S764G,S766Y	2.05E+06	8.12E-03	4.05E-09	n=4
S764G,S766Y	2.57E+06	2.50E-02	1.13E-08	n=3

EXAMPLE 5***Further Kinetic Analysis*****Method in Brief:**

[0228] Additional mutation combinations were then generated using the same experimental approaches and a detailed kinetic analysis performed.

[0229] Mouse anti-HSA antibody was immobilized on a CM5 chip using standard NHS/EDC coupling chemistry. Typically, the immobilization level was about 14,000 RU. Each Dimer mutant of D'D3-HSA was captured on a single spot in each flow cell for 2 minutes at various concentrations ranging from 0.2 - 0.7µg/ml. Capture levels ranged from 50-250RU. An adjacent spot in which anti-HSA was immobilized, but no D'D3-HSA captured was used as a reference. Capture was performed every cycle, before FVIII (CSL627) binding analysis.

[0230] CSL627 was injected at random and in duplicate over all spots in all flow cells. At neutral pH CSL627 was injected at 1, 0.5, 0.25, 0.125 and 0.06nM. The association was monitored for 5 minutes, while the dissociation was monitored for 20 minutes as well as for 1 hour at the 1nM concentration. Buffer blanks were also injected. At pH5.5 CSL627 was injected at various concentrations and time frames that best suited the interaction taking place.

[0231] After the dissociation period the surface was regenerated with a 45 second injection of 25mM Glycine pH2.6. Running buffer throughout was 10mM HEPES, 150mM NaCl, 10mM Na Citrate, 2.5mM CaCl₂, 0.1%BSA, pH7.3 and pH5.5, while the flow rate was 30 µl/min. Each interaction was measured 4 times (n=4) at 37°C.

[0232] Responses for binding to the reference spot were subtracted from those of the vWF-HSA captured spots. Responses from blank injections were then subtracted from those of all other samples to produce double-referenced sensorgrams. Double referenced sensorgrams were fitted to a 1:1 kinetic model, including a term for mass transport limitation. Association and dissociation rates were fitted globally and R_{max} fitted locally.

[0233] The results are set out in Tables 26 and 27 and Figures 4 and 5.

Table 26: Detailed Kinetics at pH 7: affinities and kinetic rates of CSL627 (Factor VIII) binding to mutant D'D3-HSA dimers.

vWF D'D3-HSA Dimer	ka (1/Ms)	kd (1/s)	KD (M)	Sample Size (n)
S764G, S766Y, V1083A	1.33E+07 ± 1.58E+06	6.44E-05 ± 3.39E-06	4.96E-12 ± 3.31E-13	n=4
S764E, S766Y, V1083A	8.59E+06 ± 4.21E+05	4.77E-05 ± 3.59E-06	5.65E-12 ± 5.82E-13	n=5
S766Y, V1083A	1.47E+07 ± 1.2E+06	8.88E-05 ± 7.15E-06	6.05E-12 ± 2.53E-13	n=4
S764E, V1083A	1.77E+07 ± 1.74E+06	2.07E-04 ± 3.04E-05	1.16E-11 ± 1.03E-12	n=4
S764G, V1083A	1.89E+07 ± 7.01E+05	3.59E-04 ± 8.59E-06	1.90E-11 ± 3.74E-13	n=4
Wildtype	2.31E+07 ± 3.03E-06	2.22E-03 ± 1.02E-04	9.88E-11 ± 9.8E-12	n=4

Table 27: Detailed Kinetics at pH5.5: affinities and kinetic rates of CSL627 binding to mutant D'D3-HSA dimers.

vWF D'D3-HSA Dimer	ka (1/Ms)	kd (1/s)	KD (M)	Sample Size (n)
S764E, S766Y, V1083A	3.61E+06 ± 2.12E+05	1.89E-03 ± 6.42E-05	5.35E-10 ± 2.64E-11	n=4
S766Y, V1083A	4.04E+06 ± 4.64E+05	9.55E-03 ± 7.47E-04	2.38E-09 ± 8.42E-11	n=3
S764G, S766Y, V1083A	6.81E+06 ± 3.77E+06	1.56E-02 ± 6.27E-03	2.93E-09 ± 4.70E-10	n=4
S764E, V1083A	2.50E+06 ± 1.67E+05	2.07E-02 ± 7.8E-04	8.32E-09 ± 3.12E-10	n=4
S764G, V1083A	9.08E+05 ± 3.54E+04	7.44E-02 ± 1.37E-03	8.24E-08 ± 4.18E-09	n=4
Wildtype	6.48E+05 ± 2.32E+04	7.91E-02 ± 9.81E-03	1.22E-07 ± 1.36E-08	n=3

EXAMPLE 6

PK analysis and impact on FVIII half-life

Methods

[0234] A stable CHO derived cell line expressing the Hu vWF D'D3-FP S764E; S766Y variant was generated using standard experimental methods. Material was produced from the stable cell line in a 10L bioreactor and vWF D'D3-FP S764E; S766Y dimer purified as previously described.

[0235] To assess the relative impact of wild-type and the vWF D'D3-FP S764E; S766Y variant on FVIII levels, CD Rats (3 animals/group) were given a combination of recombinant FVIII (CSL627 at 200IU/kg) and vWF-FP proteins at the doses shown in Table 9. Plasma samples were taken at 0, 3, 8, 24, 48, 56 and 72 hours following iv administration and FVIII levels determined using an Asserachrom FVIII:Ag ELISA. This data was then used to determine the FVIII Half-life and Mean Residence Times given in Table 28.

Table 28: PK Analysis: FVIII Half-life and Mean Residence time following co administration of recombinant FVIII and D'D3-HSA dimers

Treatment group (readout based on Asserachrom FVIII:Ag ELISA)	Mean Residence Time (hrs)	T $\frac{1}{2}$ (hrs)
CSL627 - rVIII-SingleChain, 200IU/kg	10.3	7.1
rD'D3-FP S764E; S766Y 0.09 mg/kg + CSL627 200IU/kg	13.1	9.1
rD'D3-FP S764E; S766Y 0.3 mg/kg + CSL627 200IU/kg	17.8	12.3
rD'D3-FP S764E; S766Y 0.9 mg/kg + CSL627 200IU/kg	22.6	15.6
rD'D3-FP wild type 1.0 mg/kg + CSL627 200IU/kg	14.1	10.1
rD'D3-FP wild type 3.0 mg/kg + CSL627 200IU/kg	18.4	12.7
rD'D3-FP wild type 10.0 mg/kg + CSL627 200IU/kg	26.2	18.1

Conclusion:

[0236] From the initial screen D'D3-HSA with mutations: S764P, S766W, V1083A (referred as PWA mutant) and S764G, S766Y, V1083A (referred to as GYA mutant) appeared to have the strongest affinity and slowest off rate for Factor VIII.

[0237] At neutral pH, vWF D'D3-HSA mutant dimers with the most improved affinity and off rate for CSL627 (Factor VIII) are S764G/S766Y/V1083A (GYA), S764E/S766Y/V1083A (EYA) and S766Y/V1083A (YA) with a 5pM KD and a 10^{-5} 1/s off rate. This is about a 20 fold improvement in affinity and 40 fold improvement in off rate compared to the wildtype dimer.

[0238] At acidic pH, the vWF D'D3-HSA mutant dimer with the most improved affinity and off rate for CSL627 was EYA with a 500pM KD and a 10^{-3} 1/s off rate. Based on this, the improvement in affinity and off rate for EYA is about 100 fold and at least 10 fold respectively compared to the wildtype dimer.

[0239] EYA Dimer appeared to have similar kinetic rates and affinity for CSL627 as S764P/S766I at both neutral and acidic pH.

Table 14

Mutant	ka (1/Ms)	kd (1/s)	KD (M)
S764P-L765H-S766I	1.56E+06	6.60E-05	4.24E-11
S764P-L765V-S766I	5.62E+07	1.16E-04	2.07E-12
S764P-L765M-S766I	5.69E+07	1.37E-04	2.41E-12
S764P-L765W-S766I	1.11E+06	1.46E-04	1.32E-10
S764P-L765Q-S766I	1.15E+06	2.86E-04	2.48E-10
S764P-L765K-S766I	6.88E+07	1.50E-03	2.18E-11
S764P-L765Y-S766I	5.17E+07	1.90E-03	3.67E-11
S764P-L765T-S766I	1.15E+08	3.31E-03	2.87E-11
S764P-L765I-S766I	6.34E+06	1.03E-02	1.62E-09
S764P-L765G-S766I	5.04E+07	1.22E-02	2.41E-10
S764P-L765R-S766I	7.96E+07	1.73E-02	2.18E-10
S764P-L765E-S766I	1.03E+06	5.50E-02	5.36E-08
S764P-L765F-S766I	N/D		
S764P-L765N-S766I	N/D		
S764P-L765D-S766I	N/D		
S764P-L765P-S766I	N/D		
S764P-L765S-S766I	N/D		
S764P-L765A-S766I	N/D		

N/D: Binding was present, but accurate kinetic parameters could not be determined

Table 15

Mutant	ka (1/Ms)	kd (1/s)	KD (M)
dupS764/S764P/S766I	6.23E+06	1.59E-03	2.55E-10
dupS764/S764P/S766I	1.25E+07	2.50E-03	1.99E-10
dS764-dL765-S766I			
dS764-dL765-S766Y	N/D		
delS764-S766Y	6.20E+06	2.07E-04	3.34E-11
delS764-S766W	6.60E+06	3.15E-04	4.78E-11
delS764-S766L	6.21E+06	5.85E-04	9.42E-11

Mutant	ka (1/Ms)	kd (1/s)	KD (M)
delS764-S766M	7.25E+06	7.26E-04	1.00E-10
delS764-S766I	7.09E+06	8.27E-04	1.17E-10
delS764-S766S	7.30E+06	8.46E-04	1.16E-10
N/D: Binding was present, but accurate kinetic parameters could not be determined			

Table 16

PH 5.5			
Mutant	ka (1/Ms)	kd (1/s)	KD (M)
S764P-S766W	2.77E+05	4.75E-05	1.72E-10
S764P-S766M	3.14E+05	9.16E-05	2.92E-10
S764P-S766L	4.45E+05	1.04E-04	2.34 E-10
WT	2.03E+06	3.88E-02	1.91E-08
S764P-S766I	N/D		
S764P-S766Y	N/D		
S764P-S766H	N/D		
N/D: Binding was present, but accurate kinetic parameters could not be determined			

Table 17

S766W, L809X mutants where X is one of the remaining genetic encoded amino acids, excluding cysteine			
Mutant	ka (1/Ms)	kd (1/s)	KD (M)
S766W-L809A	4.45E+06	1.15E-03	2.58E-10
S766W-L809D	4.46E+06	1.90E-03	4.25E-10
S766W-L809E	5.84E+06	1.55E-03	2.6511-10
S766W-L809F	3.26E+06	7.44E-04	2.28E-10
S766W-L809G	6.21E+06	2.26E-03	3.63E-10
S766W-L809H	2.87E+06	1.14E-03	3.97E-10
S766W-L809I	5.23E+06	5.41E-04	1.03E-10
S766W-L809K	7.00E+06	1.53E-03	2.19E-10
S766W-L809M	4.99E+06	5.81E-04	1.17E-10
S766W-L809N	6.15E+06	2.27E-03	3.69E-10
S766W-L809P	NB	NB	NB
S766W-L809Q	5.33E+06	1.13E-03	2.12E-10
S766W-L809R	6.07E+06	2.13E-03	3.52E-10
S766W-L809S	6.54E+06	1.44E-03	2.20E-10
S766W-L809T	8.72E+06	1.41E-03	1.61E-10
S766W-L809V	7.70E+06	9.40E-04	1.22E-10

S766W, L809X mutants where X is one of the remaining genetic encoded amino acids, excluding cysteine

Mutant	ka (1/Ms)	kd (1/s)	KD (M)
S766W-L809W	4.81E+06	3.12E-03	6.48E-10
S766W-E809Y	6.77E+06	3.39E-03	5.00E-10
vWF WT	4.98E+06	8.86E-04	1.78E-10

Table 18

S766W, S806X mutants where X is one of the remaining genetic encoded amino acids, excluding cysteine

Mutant	ka (1/Ms)	kd (1/s)	KD (M)
S766W-S806A	4.84E+06	3.76E-04	7.78E-11
S766W-S806D	4.20E+06	6.88E-04	1.64E-10
S766W-S806E	5.93E+06	1.29E-03	2.17E-10
S766W-S806F	NB	NB	NB
S766W-S806G	5.46E+06	1.34E-03	2.45E-10
S766W-S806H	8.90E+06	8.28E-04	9.30E-11
S766W-S806I	1.58E+06	4.47E-04	2.83E-10
S766W-S806K	N/D		
S766W-S806L	NB	NB	NB
S766W-S806M	2.05E+06	8.72E-04	4.25E-10
S766W-S806N	3.84E+06	5.85E-04	1.52E-10
S766W-S806P	4.26E+06	5.66E-04	1.33E-10
S766W-S806Q	4.33E+06	1.76E-03	4.07E-10
S766W-S806R	8.28E+06	1.07E-02	1.29E-09
S766W-S806T	5.25E+06	6.54E-04	1.25E-10
S766W-S806V	4.17E+06	6.19E-04	1.49E-10
S766W-S806W	NB	NB	NB
S766W-S806Y	NB	NB	NB
vWF WT	4.98E+06	8.86E-04	1.78E-10

N/D: Binding was present, but accurate kinetic parameters could not be determined

Table 19

S766Y, P769X mutants where X is one of the remaining genetic encoded amino acids, excluding cysteine

Mutant	ka (1/Ms)	kd (1/s)	KD (M)
S766Y-P769A	4.90E+06	5.19E-04	1.06E-10
S766Y-P769D	4.63E+06	7.63E-04	1.65E-10
S766Y-P769E	4.42E+06	4.14E-04	9.36E-11

S766Y, P769X mutants were X is one of the remaining genetic encoded amino acids, excluding cysteine

Mutant	ka (1/Ms)	kd (1/s)	KD (M)
S766Y-P769F	5.54E+06	4.27E-04	7.72E-11
S766Y-P769G	3.70E+06	7.83E-04	2.12E-10
S766Y-P769H	5.16E+06	4.17E-04	8.09E-11
S766Y-P769I	NB	NB	NB
S766Y-P769K	6.31E+06	3.83E-04	6.07E-11
S766Y-P769L	6.44E+06	5.90E-04	9.17E-11
S766Y-P769M	4.75E+06	5.11E-04	1.08E-10
S766Y-P769N	1.60E+07	5.20E-04	3.25E-11
S766Y-P769Q	NB	NB	NB
S766Y-P769R	6.55E+06	2.95E-04	4.50E-11
S766Y-P769S	4.51E+06	5.11E-04	1.13E-10
S766Y-P769T	5.11E+06	5.00E-04	9.79E-11
S766Y-P769V	6.65E+06	5.65E-04	8.49E-11
S766Y-P769W	4.77E+06	4.21E-04	8.82E-11
S766Y-P769Y	4.68E+06	3.96E-04	8.47E-11
vWF WT	4.98E+06	8.86E-04	1.78E-10

Table 20

S766Y, R768X mutants were X is one of the remaining genetic encoded amino acids, excluding cysteine

Mutant	ka (1/Ms)	kd (1/s)	KD (M)
S766Y-R768A	6.99E+06	1.48E-03	2.12E-10
S766Y-R768D	4.94E+06	4.48E-03	9.08E-10
S766Y-R768E	5.65E+06	3.22E-03	5.69E-10
S766Y-R768F	6.51E+06	1.82E-03	2.79E-10
S766Y-R768G	3.20E+06	1.02E-03	3.20E-10
S766Y-R768H	4.02E+06	6.90E-04	1.72E-10
S766Y-R768I	5.03E+06	8.99E-04	1.79E-10
S766Y-R768K	3.83E+06	4.17E-04	1.09E-10
S766Y-R768L	4.24E+06	5.48E-04	1.29E-10
S766Y-R768M	4.08E+06	8.01E-04	1.96E-10
S766Y-R768N	4.18E+06	7.98E-04	1.91E-10
S766Y-R768P	6.71E+06	1.43E-03	2.13E-10
S766Y-R768Q	3.48E+06	6.06E-04	1.74E-10

S766Y, R768X mutants where X is one of the remaining genetic encoded amino acids, excluding cysteine

Mutant	ka (1/Ms)	kd (1/s)	KD (M)
S766Y-R768S	5.33E+06	1.29E-03	2.43E-10
S766Y-R768T	5.59E+06	1.43E-03	2.56E-10
S766Y-R768V	4.51E+06	9.18E-04	2.03E-10
S766Y-R768W	4.42E+06	9.40E-04	2.13E-10
S766Y-R768Y	6.74E+06	1.87E-03	2.77E-10
vWF WT	4.98E+06	8.86E-04	1.78E-10

Table 21

Dimers Binding to FVIII (pH7.3)

Mutant	ka (1/Ms)	kd (1/s)	KD (M)
S764P-S766I	1.01E+07 ($\pm 3.41E6$)	5.00E-05 ($\pm 3.37E-6$)	3.96E-12 ($\pm 2.6E-13$)
S764P-S766W	1.24E+07 ($\pm 7.28E5$)	6.21E-05 ($\pm 2.52E-6$)	4.96E-12 ($\pm 1.9E-13$)
S766Y	1.03E+07 ($\pm 3.01E6$)	2.36E-04 ($\pm 4.27E-5$)	2.51E-11 ($\pm 3.83E-12$)
S764E-S766Y	7.75E+06 ($\pm 1.71E6$)	2.36E-04 ($\pm 2.90E-5$)	3.25E-11 ($\pm 4.57E-12$)
S764I-S766W	7.54E+06 ($\pm 5.15E5$)	2.41E-04 ($\pm 5.05E-6$)	3.25E-11 ($\pm 2.25E-12$)
S764G-S766Y	1.19E+07 ($\pm 9.1E5$)	2.63E-04 ($\pm 1.41E-5$)	2.29E-11 ($\pm 3.42E-12$)
S766Y-P769R	1.18E+07 ($\pm 4.1E5$)	2.75E-04 ($\pm 1.71E-5$)	2.32E-11 ($\pm 9.54E-13$)
S766Y-P769K	1.09E+07 ($\pm 1.37E6$)	2.85E-04 ($\pm 2.08E-5$)	2.68E-11 ($\pm 1.55E-12$)
S766W-S806A	8.88E+06 ($\pm 1.11E6$)	3.00E-04 ($\pm 1.9E-5$)	3.54E-11 ($\pm 4.37E-12$)
S764Y-S766Y	1.14E+07 ($\pm 1.71E6$)	3.34E-04 ($\pm 2.7E-5$)	3.07E-11 ($\pm 3.53E-12$)
S766Y-S769N	1.21E+07 ($\pm 1.11E6$)	3.48E-04 ($\pm 3.21E-5$)	2.89E-11 ($\pm 1.75E-12$)
S764A	1.26E+07 ($\pm 1.38E6$)	6.38E-04 ($\pm 3.24E-5$)	5.14E-11 ($\pm 2.81E-12$)
WT	1.89E+07 ($\pm 2.68E6$)	1.47E-03 ($\pm 8.92E-5$)	8.25E-11 ($\pm 7.94E-12$)

Table 22

Dimers Binding to FVIII (pH5.5)

Mutant	ka (1/Ms)	kd (1/s)	KD (M)
S764P-S766I	3.10E+06 ($\pm 3.05E5$)	1.81E-03 ($\pm 6.34E-5$)	5.98E-10 ($\pm 4.93E-11$)
S764P-S766W	3.02E+06 ($\pm 2.39E5$)	1.88E-03 ($\pm 1.78E-5$)	6.37E-10 ($\pm 5.75E-11$)
S764E-S766Y	2.43E+06 ($\pm 1.6E5$)	2.71E-03 ($\pm 9.8E-5$)	1.12E-09 ($\pm 5.29E-11$)
S764Y-S766Y	3.22E+06 ($\pm 1.24E5$)	3.45E-03 ($\pm 9.01E-5$)	1.07E-09 ($\pm 4.67E-11$)
S766Y-P769R	4.66E+06 ($\pm 1.47E5$)	6.54E-03 ($\pm 2.02E-4$)	1.40E-09 ($\pm 2.29E-11$)
S764I-S766W	3.28E+06 ($\pm 1.22E5$)	7.24E-03 ($\pm 2.89E-4$)	2.21E-09 ($\pm 5.78E-11$)
S766Y-P769K	4.14E+06 ($\pm 2.95E5$)	7.40E-03 ($\pm 3.9E-4$)	1.79E-09 ($\pm 1.27E-10$)
S766Y	3.50E+06 ($\pm 2.5E5$)	7.40E-03 ($\pm 2.12E-3$)	2.92E-09 ($\pm 1.38E-10$)

Dimers Binding to FVIII (pH5.5)			
Mutant	ka (1/Ms)	kd (1/s)	KD (M)
S766Y-S769N	2.05E+06 ($\pm 2.02E5$)	1.02E-02 ($\pm 7.84E-4$)	5.01E-09 ($\pm 2.67E-10$)
S766W-S806A	8.13E+05 ($\pm 2.83E5$)	1.40E-02 ($\pm 6.74E-4$)	1.43E-08 ($\pm 2.38E-9$)
S764G-S766Y	2.66E+06 ($\pm 4.55E5$)	1.85E-02 ($\pm 1.12E-3$)	7.53E-09 ($\pm 1.15E-9$)
S764A	2.25E+06 ($\pm 1.42E6$)	4.01E-02 ($\pm 2.54E-3$)	5.26E-08 ($\pm 3.33E-9$)
WT	1.37E+06 ($\pm 2.44E5$)	4.26E-02 ($\pm 3.9E-3$)	3.54E-08 ($\pm 2.89E-9$)

EXAMPLE 3

[0240] In an extension of the work described in PCT/AU2015/050369 further mutations and combinations of mutations were investigated with an emphasis on modifications in the D3 domain. In these experiments a recombinant form of FVIII was used. This FVIII is described in Zollner et al 2013, Thrombosis Research, 132:280-287.

Methods

[0241] Plasmid constructs encoding vWF(763-1242) -HSA and containing the single, double or triple mutations listed in Table 23 were used to generate purified vWF-HSA dimer proteins using the methods described in Example 2. Detailed kinetic analysis at pH7 was set up for the top candidates, including controls.

[0242] Mouse anti-HSA antibody was immobilized on a CM5 chip using standard NHS/EDC coupling chemistry. Typically, the immobilization level was about 14,000 RU. Each Dimer mutant of vWF-HSA was captured on a single spot in each flow cell for 2 minutes at various concentrations ranging from 0.1 - 1 μ g/ml. Capture level s ranged from 100-200RU. An adjacent spot in which anti-HSA was immobilized, but no vWF-HSA captured was used as a reference. Capture was performed every cycle, before FVIII binding analysis. In initial experiments, Factor VIII was injected at random and in duplicate over all spots and all flow cells in use at 5, 1 and 1,25nM. The results of this analysis are set out in Table 23.

Table 23: Screen at pH 7: affinities and kinetic rates of Factor VIII for various mutant D'D3-HSA dimer proteins ranked from strongest to weakest affinities.

Protein	ka (1/Ms)	kd (1/s)	KD (M)	Sample size
V1083A, S764P, S766W	6.15E+06	1.17E-04	1.90E-11	n=1
V1083A, S764G, S766Y	6.19E+06	1.86E-04	3.01E-11	n=2
N1011S,V1083A,	5.31E+06	4.30E-04	8.10E-11	n=1

Protein	ka (1/Ms)	kd (1/s)	KD (M)	Sample size
K1181E				
V1083A	5.51E+06	5.39E-04	9.78E-11	n=1
S1042T	4.39E+06	4.69E-04	1.07E-10	n=2
V805A, Q1158L	4.26E+06	6.32E-04	1.49E-10	n=2
K912E, T1088S	5.17E+06	8.00E-04	1.55E-10	n=2
L781P	4.27E+06	6.99E-04	1.64E-10	n=2
WT	4.83E+06	1.08E-03	2.23E-10	n=1
R960G	4.09E+06	1.11E-03	27.72E-10	n=2
WT=wildtype				

EXAMPLE 4***Detailed Kinetic Analysis***

[0243] Subsequent experiments were conducted where detailed kinetic analysis at pH7 was set up for the top two candidates, including controls. Factor VIII was injected at 1, 0.5, 0.25, 0.125 and 0.06nM. In a similar manner detailed kinetic analysis on the top two candidates, including controls was set up at pH5.5 where Factor VIII was injected at various concentrations that best suited the interaction.

[0244] Throughout all experiments buffer blanks were also injected over all captured proteins. The association and dissociation of Factor VIII was monitored for 3 minutes respectively during the "screening" experiment. The association of CSL627 was monitored for 5 minutes and dissociation was monitored for 20 and 60 minutes during the "detailed kinetic analysis" experiments at neutral pH. At pH 5.5 the association and dissociation of Factor VIII was monitored for various time frames that best suited the interaction.

[0245] Post the dissociation period the surface was regenerated with a 45 second injection of 25mM Glycine pH2.6. Running buffer throughout was 10mM HEPES, 150mM NaCl, 10mM Na Citrate, 2.5mM CaCl₂, 0.1%BSA, pH7.3 and pH5.5, while the flow rate was 30 μ L/min. Each interaction was measured at least 2 times (n=2) at 37°C.

[0246] Responses for binding to the reference spot were subtracted from those of the vWF-HSA captured spots. Responses from blank injections were then subtracted from those of all other samples to produce double-referenced sensorgrams. Double referenced sensorgrams were fitted to a 1:1 kinetic model, including a term for mass transport limitation. Association and dissociation rates were fitted globally and Rmax fitted locally. The results are set out in

Tables 24 and 25 and shown in Figures 1-3.

Table 24: Detailed Kinetics at pH 7: affinities and kinetic rates of Factor VIII for mutant D'D3-HSA dimers.

Protein	ka (1/Ms)	kd (1/s)	KD (M)	Sample Size
V1083A,S764P, S766W (PWA)	1.15E+07	3.84E-05	3.36E-12	n=4
V1083A,S764G, S766Y (GYA)	1.63E+07	7.30E-05	4.50E-12	n=2
S764G, S766Y (GY)	2.95E+07	3.65E-04	1.25E-11	n=3
WT*	1.06E+07	8.46E-04	8.03E-11	n=2
WT=wildtype				

Table 25: Detailed Kinetics at pH5.5: affinities and kinetic rates of Factor VIII for mutant D'D3-HSA dimers

Protein	ka (1/Ms)	kd (1/s)	KD (M)	Sample Size
V1083A,S764P,S766W	2.51E+06	8.44E-04	3.42E-10	n=4
S764P,S766W	2.94E+06	1.80E-03	6.14E-10	n=2
V1083A,S764G,S766Y	2.05E+06	8.12E-03	4.05E-09	n=4
S764G,S766Y	2.57E+06	2.50E-02	1.13E-08	n=3

EXAMPLE 5

Further Kinetic Analysis

Method in Brief:

[0247] Additional mutation combinations were then generated using the same experimental approaches and a detailed kinetic analysis performed.

[0248] Mouse anti-HSA antibody was immobilized on a CM5 chip using standard NHS/EDC coupling chemistry. Typically, the immobilization level was about 14,000 RU. Each Dimer mutant of D'D3-HSA was captured on a single spot in each flow cell for 2 minutes at various concentrations ranging from 0.2 - 0.7µg/ml. Capture levels ranged from 50-250RU. An adjacent spot in which anti-HSA was immobilized, but no D'D3-HSA captured was used as a reference. Capture was performed every cycle, before FVIII (CSL627) binding analysis.

[0249] CSL627 was injected at random and in duplicate over all spots in all flow cells. At neutral pH CSL627 was injected at 1, 0.5, 0.25, 0.125 and 0.06nM. The association was monitored for 5 minutes, while the dissociation was monitored for 20 minutes as well as for 1 hour at the 1nM concentration. Buffer blanks were also injected. At pH5.5 CSL627 was injected at various concentrations and time frames that best suited the interaction taking place.

[0250] After the dissociation period the surface was regenerated with a 45 second injection of 25mM Glycine pH2.6. Running buffer throughout was 10mM HEPES, 150mM NaCl, 10mM Na Citrate, 2.5mM CaCl₂, 0.1%BSA, pH7.3 and pH5.5, while the flow rate was 30 µl/min. Each interaction was measured 4 times (n=4) at 37°C.

[0251] Responses for binding to the reference spot were subtracted from those of the vWF-HSA captured spots. Responses from blank injections were then subtracted from those of all other samples to produce double-referenced sensorgrams. Double referenced sensorgrams were fitted to a 1:1 kinetic model, including a term for mass transport limitation. Association and dissociation rates were fitted globally and Rmax fitted locally.

[0252] The results are set out in Tables 26 and 27 and Figures 4 and 5.

Table 26: Detailed Kinetics at pH 7: affinities and kinetic rates of CSL627 (Factor VIII) binding to mutant D'D3-HSA dimers.

vWF D'D3-HSA Dimer	ka (1/Ms)	kd (1/s)	KD (M)	Sample Size (n)
S764G, S766Y, V1083A	1.33E+07 ± 1.58E+06	6.44E-05 ± 3.39E-06	4.96E-12 ± 3.31E-13	n=4
S764E, S766Y, V1083A	8.59E+06 ± 4.21E+05	4.77E-05 ± 3.59E-06	5.65E-12 ± 5.82E-13	n=5
S766Y, V1083A	1.47E+07 ± 1.2E+06	8.88E-05 ± 7.15E-06	6.05E-12 ± 2.53E-13	n=4
S764E, V1083A	1.77E+07 ± 1.74E+06	2.07E-04 ± 3.04E-05	1.16E-11 ± 1.03E-12	n=4
S764G, V1083A	1.89E+07 ± 7.01E+05	3.59E-04 ± 8.59E-06	1.90E-11 ± 3.74E-13	n=4
Wildtype	2.31E+07 ± 3.03E-06	2.22E-03 ± 1.02E-04	9.88E-11 ± 9.8E-12	n=4

Table 28: Detailed Kinetics at pH5.5: affinities and kinetic rates of CSL627 binding to mutant D'D3-HSA dimers.

vWF D'D3-HSA Dimer	ka (1/Ms)	kd (1/s)	KD (M)	Sample Size (n)
S764E, S766Y, V1083A	3.61E+06 ± 2.12E+05	1.89E-03 ± 6.42E-05	5.35E-10 ± 2.64E-11	n=4
S766Y, V1083A	4.04E+06 ± 4.64E+05	9.55E-03 ± 7.47E-04	2.38E-09 ± 8.42E-11	n=3
S764G, S766Y,	6.81E+06 ±	1.56E-02 ±	2.93E-09 ±	n=4

vWF D'D3-HSA Dimer	ka (1/Ms)	kd (1/s)	KD (M)	Sample Size (n)
V1083A	3.77E+06	6.27E-03	4.70E-10	
S764E, V1083A	2.50E+06 ± 1.67E+05	2.07E-02 ± 7.8E-04	8.32E-09 ± 3.12E-10	n=4
S764G, V1083A	9.08E+05 ± 3.54E+04	7.44E-02 ± 1.37E-03	8.24E-08 ± 4.18E-09	n=4
Wildtype	6.48E+05 ± 2.32E+04	7.91E-02 ± 9.81E-03	1.22E-07 ± 1.36E-08	n=3

EXAMPLE 6***PK analysis and impact on FVIII half-life*****Methods**

[0253] A stable CHO derived cell line expressing the Hu vWF D'D3-FP S764E; S766Y variant was generated using standard experimental methods. Material was produced from the stable cell line in a 10L bioreactor and vWF D'D3-FP S764E; S766Y dimer purified as previously described.

[0254] To assess the relative impact of wild-type and the vWF D'D3-FP S764E; S766Y variant on FVIII levels, CD Rats (3 animals/group) were given a combination of recombinant FVIII (CSL627 at 200IU/kg) and vWF-FP proteins at the doses shown in Table 9. Plasma samples were taken at 0, 3, 8, 24, 48, 56 and 72 hours following iv administration and FVIII levels determined using an Asserachrom FVIII:Ag ELISA. This data was then used to determine the FVIII Half-life and Mean Residence Times given in Table 29.

Table 29: PK Analysis: FVIII Half-life and Mean Residence time following co administration of recombinant FVIII and D'D3-HSA dimers

Treatment group (readout based on Asserachrom FVIII:Ag ELISA)	Mean Residence Time (hrs)	T ½ (hrs)
CSL627 - rVIII-SingleChain, 200IU/kg	10.3	7.1
rD'D3-FP S764E; S766Y 0.09 mg/kg + CSL627 200IU/kg	13.1	9.1
rD'D3-FP S764E; S766Y 0.3 mg/kg + CSL627 200IU/kg	17.8	12.3
rD'D3-FP S764E; S766Y 0.9 mg/kg	22.6	15.6

Treatment group (readout based on Asserachrom FVIII:Ag ELISA)	Mean Residence Time (hrs)	T ½ (hrs)
+ CSL627 200IU/kg		
rD'D3-FP wild type 1.0 mg/kg + CSL627 200IU/kg	14.1	10.1
rD'D3-FP wild type 3.0 mg/kg + CSL627 200IU/kg	18.4	12.7
rD'D3-FP wild type 10.0 mg/kg + CSL627 200IU/kg	26.2	18.1

Conclusion:

[0255] From the initial screen D'D3-HSA with mutations: S764P, S766W, V1083A (referred as PWA mutant) and S764G, S766Y, V1083A (referred to as GYA mutant) appeared to have the strongest affinity and slowest off rate for Factor VIII.

[0256] At neutral pH, vWF D'D3-HSA mutant dimers with the most improved affinity and off rate for CSL627 (Factor VIII) are S764G/S766Y/V1083A (GYA), S764E/S766Y/V1083A (EYA) and S766Y/V1083A (YA) with a 5pM KD and a 10^{-5} 1/s off rate. This is about a 20 fold improvement in affinity and 40 fold improvement in off rate compared to the wildtype dimer.

[0257] At acidic pH, the vWF D'D3-HSA mutant dimer with the most improved affinity and off rate for CSL627 was EYA with a 500pM KD and a 10^{-3} 1/s off rate. Based on this, the improvement in affinity and off rate for EYA is about 100 fold and at least 10 fold respectively compared to the wildtype dimer.

[0258] EYA Dimer appeared to have similar kinetic rates and affinity for CSL627 as S764P/S766I at both neutral and acidic pH.

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

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

Cys Ala Gly Gly Leu Glu Cys Ala Cys Pro Ala 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 Ser Pro Val Cys Pro Ala Gly Met Glu Tyr Arg Gln Cys
 290 295 300
 Val Ser Pro Cys Ala Arg Thr Cys Gln Ser Leu His Ile Asn Glu Met
 305 310 315 320
 Cys Gln Glu Arg Cys Val Asp Gly Cys Ser Cys Pro Glu Gly Gln Leu
 325 330 335
 Leu Asp Glu Gly Leu Cys Val Glu Ser Thr Glu Cys Pro Cys Val His
 340 345 350
 Ser Gly Lys Arg Tyr Pro Pro Gly Thr Ser Leu Ser Arg Asp Cys Asn
 355 360 365
 Thr Cys Ile Cys Arg Asn Ser Gln 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 Gln Asp His Ser Phe Ser Ile Val Ile Glu Thr Val Gln Cys
 420 425 430
 Ala Asp Asp Arg Asp Ala Val Cys Thr Arg Ser Val Thr Val Arg Leu
 435 440 445
 Pro Gly Leu His Asn Ser Leu Val Lys Leu Lys His Gly Ala Gly Val
 450 455 460
 Ala Met Asp Gly Gln Asp Val Gln Leu Pro Leu Leu Lys Gly Asp Leu
 465 470 475 480
 Arg Ile Gln His Thr Val Thr Ala Ser Val Arg Leu Ser Tyr Gly Glu
 485 490 495
 Asp Leu Gln Met Asp Trp Asp Gly Arg Gly Arg Leu Leu Val Lys Leu
 500 505 510
 Ser Pro Val Tyr Ala Gly Lys Thr Cys Gly Leu Cys Gly Asn Tyr Asn
 515 520 525
 Gly Asn Gln Gly Asp Asp Phe Leu Thr Pro Ser Gly Leu Ala Glu Pro
 530 535 540
 Arg Val Glu Asp Phe Gly Asn Ala Trp Lys Leu His Gly Asp Cys Gln
 545 550 555 560
 Asp Leu Gln Lys Gln His Ser Asp Pro Cys Ala Leu Asn Pro Arg Met
 565 570 575

Thr Arg Phe Ser Glu Glu Ala Cys Ala Val Leu Thr Ser Pro Thr Phe
580 585 590

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

Arg Tyr Asp Val Cys Ser Cys Ser Asp Gly Arg Glu Cys Leu Cys Gly
610 615 620

Ala Leu Ala Ser Tyr Ala Ala Ala Cys Ala Gly Arg Gly Val Arg Val
625 630 635 640

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

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

Ser Tyr Pro Asp Glu Glu Cys Asn Glu Ala Cys Leu Glu Gly Cys Phe
675 680 685

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

Ala Gln Cys Pro Cys Tyr Tyr Asp Gly Glu Ile Phe Gln Pro Glu 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 Met Ser Gly Val Pro Gly Ser Leu Leu Pro Asp Ala 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 Leu Arg Ala Glu
770 775 780

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

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

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

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

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

Ala Thr Cys Ser Thr Ile Glu Met Ala His Thr Leu Thr Phe Asp Glu

Ala Thr Cys Ser Thr 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

Tyr Cys Gly Ser Asn Pro Gly Thr Phe Arg Ile Leu Val Gly Asn Lys
 900 905 910

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

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

Arg Pro Met Lys Asp Glu Thr His Phe Glu Val Val Glu Ser Gly Arg
 945 950 955 960

Tyr Ile Ile Leu Leu Leu Gly Lys Ala Leu Ser Val Val Trp Asp Arg
 965 970 975

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

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

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

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
 1040 1045 1050

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

Gln Asp Cys Asn Lys Leu Val Asp Pro Glu Pro Tyr Leu Asp Val
 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 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 1160	Pro	Glu	Pro	Leu	Ala	Cys 1165	Pro	Val	Gln	Cys	Val 1170	Glu	Gly	Cys	
His 1175	Ala		His	Cys	Pro	Pro	Gly 1180	Lys	Ile	Leu	Asp	Glu 1185	Leu	Leu	Gln
Thr 1190	Cys	Val	Asp	Pro	Glu	Asp 1195	Cys	Pro	Val	Cys	Glu 1200	Val	Ala	Gly	
Arg 1205	Arg	Phe	Ala	Ser	Gly	Lys 1210	Lys	Val	Thr	Leu	Asn 1215	Pro	Ser	Asp	
Pro 1220	Glu	His	Cys	Gln	Ile	Cys 1225	His	Cys	Asp	Val	Val 1230	Asn	Leu	Thr	
Cys 1235	Glu	Ala	Cys	Gln	Glu	Pro 1240	Gly	Gly	Leu	Val	Val 1245	Pro	Pro	Thr	
Asp 1250	Ala	Pro	Val	Ser	Pro	Thr 1255	Thr	Leu	Tyr	Val	Glu 1260	Asp	Ile	Ser	
Glu 1265	Pro	Pro	Leu	His	Asp	Phe 1270	Tyr	Cys	Ser	Arg	Leu 1275	Leu	Asp	Leu	
Val 1280	Phe	Leu	Leu	Asp	Gly	Ser 1285	Ser	Arg	Leu	Ser	Glu 1290	Ala	Glu	Phe	
Glu 1295	Val	Leu	Lys	Ala	Phe	Val 1300	Val	Asp	Met	Met	Glu 1305	Arg	Leu	Arg	
Ile 1310	Ser	Gln	Lys	Trp	Val	Arg 1315	Val	Ala	Val	Val	Glu 1320	Tyr	His	Asp	
Gly 1325	Ser	His	Ala	Tyr	Ile	Gly 1330	Leu	Lys	Asp	Arg	Lys 1335	Arg	Pro	Ser	
Glu 1340	Leu	Arg	Arg	Ile	Ala	Ser 1345	Gln	Val	Lys	Tyr	Ala 1350	Gly	Ser	Gln	
Val 1355	Ala	Ser	Thr	Ser	Glu	Val 1360	Leu	Lys	Tyr	Thr	Leu 1365	Phe	Gln	Ile	
Phe 1370	Ser	Lys	Ile	Asp	Arg	Pro 1375	Glu	Ala	Ser	Arg	Ile 1380	Thr	Leu	Leu	
Leu 1385	Met	Ala	Ser	Gln	Glu	Pro 1390	Gln	Arg	Met	Ser	Arg 1395	Asn	Phe	Val	
Arg 1400	Tyr	Val	Gln	Gly	Leu	Lys 1405	Lys	Lys	Lys	Val	Ile 1410	Val	Ile	Pro	
Val 1415	Gly	Ile	Gly	Pro	His	Ala 1420	Asn	Leu	Lys	Gln	Ile 1425	Arg	Leu	Ile	
Glu 1430	Lys	Gln	Ala	Pro	Glu	Asn 1435	Lys	Ala	Phe	Val	Leu 1440	Ser	Ser	Val	
Asp	Glu	Leu	Glu	Gln	Gln	Arg	Asp	Glu	Ile	Val	Ser	Tyr	Leu	Cys	

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1445              1450              1455  - - - - -

Asp Leu  Ala Pro Glu Ala Pro  Pro Pro Thr Leu Pro  Pro Asp 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
1640              1645              1650

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

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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	
1790			1795			1800			
Ser Val	Asp Ser	Val Asp	Ala	Ala Ala	Asp Ala	Ala	Arg Ser	Asn	
1805			1810			1815			
Arg Val	Thr Val	Phe Pro	Ile	Gly Ile	Gly Asp	Arg	Tyr Asp	Ala	
1820			1825			1830			
Ala Gln	Leu Arg	Ile Leu	Ala	Gly Pro	Ala Gly	Asp	Ser Asn	Val	
1835			1840			1845			
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 2015	Glu	Leu	His	Ser	Asp	Met 2020	Glu	Val	Thr	Val	Asn 2025	Gly	Arg	Leu
Val 2030	Ser	Val	Pro	Tyr	Val	Gly 2035	Gly	Asn	Met	Glu	Val 2040	Asn	Val	Tyr
Gly 2045	Ala	Ile	Met	His	Glu	Val 2050	Arg	Phe	Asn	His	Leu 2055	Gly	His	Ile
Phe 2060	Thr	Phe	Thr	Pro	Gln	Asn 2065	Asn	Glu	Phe	Gln	Leu 2070	Gln	Leu	Ser
Pro 2075	Lys	Thr	Phe	Ala	Ser	Lys 2080	Thr	Tyr	Gly	Leu	Cys 2085	Gly	Ile	Cys
Asp 2090	Glu	Asn	Gly	Ala	Asn	Asp 2095	Phe	Met	Leu	Arg	Asp 2100	Gly	Thr	Val
Thr 2105	Thr	Asp	Trp	Lys	Thr	Leu 2110	Val	Gln	Glu	Trp	Thr 2115	Val	Gln	Arg
Pro 2120	Gly	Gln	Thr	Cys	Gln	Pro 2125	Ile	Leu	Glu	Glu	Gln 2130	Cys	Leu	Val
Pro 2135	Asp	Ser	Ser	His	Cys	Gln 2140	Val	Leu	Leu	Leu	Pro 2145	Leu	Phe	Ala
Glu 2150	Cys	His	Lys	Val	Leu	Ala 2155	Pro	Ala	Thr	Phe	Tyr 2160	Ala	Ile	Cys
Gln 2165	Gln	Asp	Ser	Cys	His	Gln 2170	Glu	Gln	Val	Cys	Glu 2175	Val	Ile	Ala
Ser 2180	Tyr	Ala	His	Leu	Cys	Arg 2185	Thr	Asn	Gly	Val	Cys 2190	Val	Asp	Trp
Arg 2195	Thr	Pro	Asp	Phe	Cys	Ala 2200	Met	Ser	Cys	Pro	Pro 2205	Ser	Leu	Val
Tyr 2210	Asn	His	Cys	Glu	His	Gly 2215	Cys	Pro	Arg	His	Cys 2220	Asp	Gly	Asn
Val 2225	Ser	Ser	Cys	Gly	Asp	His 2230	Pro	Ser	Glu	Gly	Cys 2235	Phe	Cys	Pro
Pro 2240	Asp	Lys	Val	Met	Leu	Glu 2245	Gly	Ser	Cys	Val	Pro 2250	Glu	Glu	Ala
Cys 2255	Thr	Gln	Cys	Ile	Gly	Glu 2260	Asp	Gly	Val	Gln	His 2265	Gln	Phe	Leu
Glu 2270	Ala	Trp	Val	Pro	Asp	His 2275	Gln	Pro	Cys	Gln	Ile 2280	Cys	Thr	Cys
Leu 2285	Ser	Gly	Arg	Lys	Val	Asn 2290	Cys	Thr	Thr	Gln	Pro 2295	Cys	Pro	Thr

Ala Lys 2300	Ala Pro Thr Cys Gly 2305	Leu Cys Glu Val 2310	Ala Arg Leu Arg
Gln Asn 2315	Ala Asp Gln Cys Cys 2320	Pro Glu Tyr Glu Cys 2325	Val Cys Asp
Pro Val 2330	Ser Cys Asp Leu Pro 2335	Pro Val Pro His Cys 2340	Glu Arg Gly
Leu Gln 2345	Pro Thr Leu Thr Asn 2350	Pro Gly Glu Cys Arg 2355	Pro Asn Phe
Thr Cys 2360	Ala Cys Arg Lys Glu 2365	Glu Cys Lys Arg Val 2370	Ser Pro Pro
Ser Cys 2375	Pro Pro His Arg Leu 2380	Pro Thr Leu Arg Lys 2385	Thr Gln Cys
Cys Asp 2390	Glu Tyr Glu Cys Ala 2395	Cys Asn Cys Val Asn 2400	Ser Thr Val
Ser Cys 2405	Pro Leu Gly Tyr Leu 2410	Ala Ser Thr Ala Thr 2415	Asn Asp Cys
Gly Cys 2420	Thr Thr Thr Thr Cys 2425	Leu Pro Asp Lys Val 2430	Cys Val His
Arg Ser 2435	Thr Ile Tyr Pro Val 2440	Gly Gln Phe Trp Glu 2445	Glu Gly Cys
Asp Val 2450	Cys Thr Cys Thr Asp 2455	Met Glu Asp Ala Val 2460	Met Gly Leu
Arg Val 2465	Ala Gln Cys Ser Gln 2470	Lys Pro Cys Glu Asp 2475	Ser Cys Arg
Ser Gly 2480	Phe Thr Tyr Val Leu 2485	His Glu Gly Glu Cys 2490	Cys Gly Arg
Cys Leu 2495	Pro Ser Ala Cys Glu 2500	Val Val Thr Gly Ser 2505	Pro Arg Gly
Asp Ser 2510	Gln Ser Ser Trp Lys 2515	Ser Val Gly Ser Gln 2520	Trp Ala Ser
Pro Glu 2525	Asn Pro Cys Leu Ile 2530	Asn Glu Cys Val Arg 2535	Val Lys Glu
Glu Val 2540	Phe Ile Gln Gln Arg 2545	Asn Val Ser Cys Pro 2550	Gln Leu Glu
Val Pro 2555	Val Cys Pro Ser Gly 2560	Phe Gln Leu Ser Cys 2565	Lys Thr Ser
Ala Cys 2570	Cys Pro Ser Cys Arg 2575	Cys Glu Arg Met Glu 2580	Ala Cys Met

Leu Asn Gly Thr Val Ile Gly Pro Gly Lys Thr Val Met Ile Asp
 2585 2590 2595
 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
 2780 2785 2790
 Val Tyr His Glu Val Leu Asn Ala Met Glu Cys Lys Cys Ser Pro
 2795 2800 2805
 Arg Lys Cys Ser Lys
 2810

<210> 3

<211> 479

<212> PRT

<213> Homo sapiens

<400> 3

Ser Leu Ser Cys Arg Pro Pro Met Val Lys Leu Val Cys Pro Ala Asp
 1 5 10 15

Asn Leu Arg Ala Glu Gly Leu Glu Cys Thr Lys Thr Cys Gln Asn Tyr
 20 25 30

Asp Leu Glu Cys Met Ser Met Gly Cys Val Ser Gly Cys Leu Cys Pro
 35 40 45

Pro Gly Met Val Arg His Glu Asn Arg Cys Val Ala Leu Glu Arg Cys
 50 55 60

Pro Cys Phe His Gln Gly Lys Glu Tyr Ala Pro Gly Glu Thr Val Lys

65 70 75 80

Ile Gly Cys Asn Thr Cys Val Cys Arg Asp Arg Lys Trp Asn Cys Thr
 85 90 95

Asp His Val Cys Asp Ala Thr Cys Ser Thr Ile Gly Met Ala His Tyr
 100 105 110

Leu Thr Phe Asp Gly Leu Lys Tyr Leu Phe Pro Gly Glu Cys Gln Tyr
 115 120 125

Val Leu Val Gln Asp Tyr Cys Gly Ser Asn Pro Gly Thr Phe Arg Ile
 130 135 140

Leu Val Gly Asn Lys Gly Cys Ser His Pro Ser Val Lys Cys Lys Lys
 145 150 155 160

Arg Val Thr Ile Leu Val Glu Gly Gly Glu Ile Glu Leu Phe Asp Gly
 165 170 175

Glu Val Asn Val Lys Arg Pro Met Lys Asp Glu Thr His Phe Glu Val
 180 185 190

Val Glu Ser Gly Arg Tyr Ile Ile Leu Leu Leu Gly Lys Ala Leu Ser
 195 200 205

Val Val Trp Asp Arg His Leu Ser Ile Ser Val Val Leu Lys Gln Thr
 210 215 220

Tyr Gln Glu Lys Val Cys Gly Leu Cys Gly Asn Phe Asp Gly Ile Gln
 225 230 235 240

Asn Asn Asp Leu Thr Ser Ser Asn Leu Gln Val Glu Glu Asp Pro Val
 245 250 255

Asp Phe Gly Asn Ser Trp Lys Val Ser Ser Gln Cys Ala Asp Thr Arg
 260 265 270

Lys Val Pro Leu Asp Ser Ser Pro Ala Thr Cys His Asn Asn Ile Met
 275 280 285

Lys Gln Thr Met Val Asp Ser Ser Cys Arg Ile Leu Thr Ser Asp Val
290 295 300

Phe Gln Asp Cys Asn Lys Leu Val Asp Pro Glu Pro Tyr Leu Asp Val
305 310 315 320

Cys Ile Tyr Asp Thr Cys Ser Cys Glu Ser Ile Gly Asp Cys Ala Cys
325 330 335

Phe Cys Asp Thr Ile Ala Ala Tyr Ala His Val Cys Ala Gln His Gly
340 345 350

Lys Val Val Thr Trp Arg Thr Ala Thr Leu Cys Pro Gln Ser Cys Glu
355 360 365

Glu Arg Asn Leu Arg Glu Asn Gly Tyr Glu Cys Glu Trp Arg Tyr Asn
370 375 380

Ser Cys Ala Pro Ala Cys Gln Val Thr Cys Gln His Pro Glu Pro Leu
385 390 395 400

Ala Cys Pro Val Gln Cys Val Glu Gly Cys His Ala His Cys Pro Pro
405 410 415

Gly Lys Ile Leu Asp Glu Leu Leu Gln Thr Cys Val Asp Pro Glu Asp
420 425 430

Cys Pro Val Cys Glu Val Ala Gly Arg Arg Phe Ala Ser Gly Lys Lys
435 440 445

Val Thr Leu Asn Pro Ser Asp Pro Glu His Cys Gln Ile Cys His Cys
450 455 460

Asp Val Val Asn Leu Thr Cys Glu Ala Cys Gln Glu Pro Gly Gly
465 470 475

<210> 4

<211> 2050

<212> PRT

<213> Homo sapiens

<400> 4

Ser Leu Ser Cys Arg Pro Pro Met Val Lys Leu Val Cys Pro Ala Asp
1 5 10 15

Asn Leu Arg Ala Glu Gly Leu Glu Cys Thr Lys Thr Cys Gln Asn Tyr
20 25 30

Asp Leu Glu Cys Met Ser Met Gly Cys Val Ser Gly Cys Leu Cys Pro
35 40 45

Pro Gly Met Val Arg His Glu Asn Arg Cys Val Ala Leu Glu Arg Cys
50 55 60

Pro Cys Phe His Gln Gly Lys Glu Tyr Ala Pro Gly Glu Thr Val Lys
65 70 75 80

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      55              70              75              80
Ile Gly Cys Asn Thr Cys Val Cys Arg Asp Arg Lys Trp Asn Cys Thr
      85              90              95

Asp His Val Cys Asp Ala Thr Cys Ser Thr Ile Gly Met Ala His Tyr
      100             105             110

Leu Thr Phe Asp Gly Leu Lys Tyr Leu Phe Pro Gly Glu Cys Gln Tyr
      115             120             125

Val Leu Val Gln Asp Tyr Cys Gly Ser Asn Pro Gly Thr Phe Arg Ile
      130             135             140

Leu Val Gly Asn Lys Gly Cys Ser His Pro Ser Val Lys Cys Lys Lys
      145             150             155             160

Arg Val Thr Ile Leu Val Glu Gly Gly Glu Ile Glu Leu Phe Asp Gly
      165             170             175

Glu Val Asn Val Lys Arg Pro Met Lys Asp Glu Thr His Phe Glu Val
      180             185             190

Val Glu Ser Gly Arg Tyr Ile Ile Leu Leu Leu Gly Lys Ala Leu Ser
      195             200             205

Val Val Trp Asp Arg His Leu Ser Ile Ser Val Val Leu Lys Gln Thr
      210             215             220

Tyr Gln Glu Lys Val Cys Gly Leu Cys Gly Asn Phe Asp Gly Ile Gln
      225             230             235             240

Asn Asn Asp Leu Thr Ser Ser Asn Leu Gln Val Glu Glu Asp Pro Val
      245             250             255

Asp Phe Gly Asn Ser Trp Lys Val Ser Ser Gln Cys Ala Asp Thr Arg
      260             265             270

Lys Val Pro Leu Asp Ser Ser Pro Ala Thr Cys His Asn Asn Ile Met
      275             280             285

Lys Gln Thr Met Val Asp Ser Ser Cys Arg Ile Leu Thr Ser Asp Val
      290             295             300

Phe Gln Asp Cys Asn Lys Leu Val Asp Pro Glu Pro Tyr Leu Asp Val
      305             310             315             320

Cys Ile Tyr Asp Thr Cys Ser Cys Glu Ser Ile Gly Asp Cys Ala Cys
      325             330             335

Phe Cys Asp Thr Ile Ala Ala Tyr Ala His Val Cys Ala Gln His Gly
      340             345             350

Lys Val Val Thr Trp Arg Thr Ala Thr Leu Cys Pro Gln Ser Cys Glu
      355             360             365

Glu Arg Asn Leu Arg Glu Asn Gly Tyr Glu Cys Glu Trp Arg Tyr Asn
      370             375             380

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Ser Cys Ala Pro Ala Cys Gln Val Thr Cys Gln His Pro Glu Pro Leu
 385 390 395 400
 Ala Cys Pro Val Gln Cys Val Glu Gly Cys His Ala His Cys Pro Pro
 405 410 415
 Gly Lys Ile Leu Asp Glu Leu Leu Gln Thr Cys Val Asp Pro Glu Asp
 420 425 430
 Cys Pro Val Cys Glu Val Ala Gly Arg Arg Phe Ala Ser Gly Lys Lys
 435 440 445
 Val Thr Leu Asn Pro Ser Asp Pro Glu His Cys Gln Ile Cys His Cys
 450 455 460
 Asp Val Val Asn Leu Thr Cys Glu Ala Cys Gln Glu Pro Gly Gly Leu
 465 470 475 480
 Val Val Pro Pro Thr Asp Ala Pro Val Ser Pro Thr Thr Leu Tyr Val
 485 490 495
 Glu Asp Ile Ser Glu Pro Pro Leu His Asp Phe Tyr Cys Ser Arg Leu
 500 505 510
 Leu Asp Leu Val Phe Leu Leu Asp Gly Ser Ser Arg Leu Ser Glu Ala
 515 520 525
 Glu Phe Glu Val Leu Lys Ala Phe Val Val Asp Met Met Glu Arg Leu
 530 535 540
 Arg Ile Ser Gln Lys Trp Val Arg Val Ala Val Val Glu Tyr His Asp
 545 550 555 560
 Gly Ser His Ala Tyr Ile Gly Leu Lys Asp Arg Lys Arg Pro Ser Glu
 565 570 575
 Leu Arg Arg Ile Ala Ser Gln Val Lys Tyr Ala Gly Ser Gln Val Ala
 580 585 590
 Ser Thr Ser Glu Val Leu Lys Tyr Thr Leu Phe Gln Ile Phe Ser Lys
 595 600 605
 Ile Asp Arg Pro Glu Ala Ser Arg Ile Thr Leu Leu Leu Met Ala Ser
 610 615 620
 Gln Glu Pro Gln Arg Met Ser Arg Asn Phe Val Arg Tyr Val Gln Gly
 625 630 635 640
 Leu Lys Lys Lys Lys Val Ile Val Ile Pro Val Gly Ile Gly Pro His
 645 650 655
 Ala Asn Leu Lys Gln Ile Arg Leu Ile Glu Lys Gln Ala Pro Glu Asn
 660 665 670
 Lys Ala Phe Val Leu Ser Ser Val Asp Glu Leu Glu Gln Gln Arg Asp
 675 680 685

Glu Ile Val Ser Tyr Leu Cys Asp Leu Ala Pro Glu Ala Pro Pro Pro
690 695 700

Thr Leu Pro Pro Asp Met Ala Gln Val Thr Val Gly Pro Gly Leu Leu
705 710 715 720

Gly Val Ser Thr Leu Gly Pro Lys Arg Asn Ser Met Val Leu Asp Val
725 730 735

Ala Phe Val Leu Glu Gly Ser Asp Lys Ile Gly Glu Ala Asp Phe Asn
740 745 750

Arg Ser Lys Glu Phe Met Glu Glu Val Ile Gln Arg Met Asp Val Gly
755 760 765

Gln Asp Ser Ile His Val Thr Val Leu Gln Tyr Ser Tyr Met Val Thr
770 775 780

Val Glu Tyr Pro Phe Ser Glu Ala Gln Ser Lys Gly Asp Ile Leu Gln
785 790 795 800

Arg Val Arg Glu Ile Arg Tyr Gln Gly Gly Asn Arg Thr Asn Thr Gly
805 810 815

Leu Ala Leu Arg Tyr Leu Ser Asp His Ser Phe Leu Val Ser Gln Gly
820 825 830

Asp Arg Glu Gln Ala Pro Asn Leu Val Tyr Met Val Thr Gly Asn Pro
835 840 845

Ala Ser Asp Glu Ile Lys Arg Leu Pro Gly Asp Ile Gln Val Val Pro
850 855 860

Ile Gly Val Gly Pro Asn Ala Asn Val Gln Glu Leu Glu Arg Ile Gly
865 870 875 880

Trp Pro Asn Ala Pro Ile Leu Ile Gln Asp Phe Glu Thr Leu Pro Arg
885 890 895

Glu Ala Pro Asp Leu Val Leu Gln Arg Cys Cys Ser Gly Glu Gly Leu
900 905 910

Gln Ile Pro Thr Leu Ser Pro Ala Pro Asp Cys Ser Gln Pro Leu Asp
915 920 925

Val Ile Leu Leu Leu Asp Gly Ser Ser Ser Phe Pro Ala Ser Tyr Phe
930 935 940

Asp Glu Met Lys Ser Phe Ala Lys Ala Phe Ile Ser Lys Ala Asn Ile
945 950 955 960

Gly Pro Arg Leu Thr Gln Val Ser Val Leu Gln Tyr Gly Ser Ile Thr
965 970 975

Thr Ile Asp Val Pro Trp Asn Val Val Pro Glu Lys Ala His Leu Leu
 980 985 990

Ser Leu Val Asp Val Met Gln Arg Glu Gly Gly Pro Ser Gln Ile Gly
 995 1000 1005

Asp Ala Leu Gly Phe Ala Val Arg Tyr Leu Thr Ser Glu Met His
 1010 1015 1020

Gly Ala Arg Pro Gly Ala Ser Lys Ala Val Val Ile Leu Val Thr
 1025 1030 1035

Asp Val Ser Val Asp Ser Val Asp Ala Ala Ala Asp Ala Ala Arg
 1040 1045 1050

Ser Asn Arg Val Thr Val Phe Pro Ile Gly Ile Gly Asp Arg Tyr
 1055 1060 1065

Asp Ala Ala Gln Leu Arg Ile Leu Ala Gly Pro Ala Gly Asp Ser
 1070 1075 1080

Asn Val Val Lys Leu Gln Arg Ile Glu Asp Leu Pro Thr Met Val
 1085 1090 1095

Thr Leu Gly Asn Ser Phe Leu His Lys Leu Cys Ser Gly Phe Val
 1100 1105 1110

Arg Ile Cys Met Asp Glu Asp Gly Asn Glu Lys Arg Pro Gly Asp
 1115 1120 1125

Val Trp Thr Leu Pro Asp Gln Cys His Thr Val Thr Cys Gln Pro
 1130 1135 1140

Asp Gly Gln Thr Leu Leu Lys Ser His Arg Val Asn Cys Asp Arg
 1145 1150 1155

Gly Leu Arg Pro Ser Cys Pro Asn Ser Gln Ser Pro Val Lys Val
 1160 1165 1170

Glu Glu Thr Cys Gly Cys Arg Trp Thr Cys Pro Cys Val Cys Thr
 1175 1180 1185

Gly Ser Ser Thr Arg His Ile Val Thr Phe Asp Gly Gln Asn Phe
 1190 1195 1200

Lys Leu Thr Gly Ser Cys Ser Tyr Val Leu Phe Gln Asn Lys Glu
 1205 1210 1215

Gln Asp Leu Glu Val Ile Leu His Asn Gly Ala Cys Ser Pro Gly
 1220 1225 1230

Ala Arg Gln Gly Cys Met Lys Ser Ile Glu Val Lys His Ser Ala
 1235 1240 1245

Leu Ser Val Glu Leu His Ser Asp Met Glu Val Thr Val Asn Gly
 1250 1255 1260

Arg	Leu	Val	Ser	Val	Pro	Tyr	Val	Gly	Gly	Asn	Met	Glu	Val	Asn
	1265					1270					1275			
Val	Tyr	Gly	Ala	Ile	Met	His	Glu	Val	Arg	Phe	Asn	His	Leu	Gly
	1280					1285					1290			
His	Ile	Phe	Thr	Phe	Thr	Pro	Gln	Asn	Asn	Glu	Phe	Gln	Leu	Gln
	1295					1300					1305			
Leu	Ser	Pro	Lys	Thr	Phe	Ala	Ser	Lys	Thr	Tyr	Gly	Leu	Cys	Gly
	1310					1315					1320			
Ile	Cys	Asp	Glu	Asn	Gly	Ala	Asn	Asp	Phe	Met	Leu	Arg	Asp	Gly
	1325					1330					1335			
Thr	Val	Thr	Thr	Asp	Trp	Lys	Thr	Leu	Val	Gln	Glu	Trp	Thr	Val
	1340					1345					1350			
Gln	Arg	Pro	Gly	Gln	Thr	Cys	Gln	Pro	Ile	Leu	Glu	Glu	Gln	Cys
	1355					1360					1365			
Leu	Val	Pro	Asp	Ser	Ser	His	Cys	Gln	Val	Leu	Leu	Leu	Pro	Leu
	1370					1375					1380			
Phe	Ala	Glu	Cys	His	Lys	Val	Leu	Ala	Pro	Ala	Thr	Phe	Tyr	Ala
	1385					1390					1395			
Ile	Cys	Gln	Gln	Asp	Ser	Cys	His	Gln	Glu	Gln	Val	Cys	Glu	Val
	1400					1405					1410			
Ile	Ala	Ser	Tyr	Ala	His	Leu	Cys	Arg	Thr	Asn	Gly	Val	Cys	Val
	1415					1420					1425			
Asp	Trp	Arg	Thr	Pro	Asp	Phe	Cys	Ala	Met	Ser	Cys	Pro	Pro	Ser
	1430					1435					1440			
Leu	Val	Tyr	Asn	His	Cys	Glu	His	Gly	Cys	Pro	Arg	His	Cys	Asp
	1445					1450					1455			
Gly	Asn	Val	Ser	Ser	Cys	Gly	Asp	His	Pro	Ser	Glu	Gly	Cys	Phe
	1460					1465					1470			
Cys	Pro	Pro	Asp	Lys	Val	Met	Leu	Glu	Gly	Ser	Cys	Val	Pro	Glu
	1475					1480					1485			
Glu	Ala	Cys	Thr	Gln	Cys	Ile	Gly	Glu	Asp	Gly	Val	Gln	His	Gln
	1490					1495					1500			
Phe	Leu	Glu	Ala	Trp	Val	Pro	Asp	His	Gln	Pro	Cys	Gln	Ile	Cys
	1505					1510					1515			
Thr	Cys	Leu	Ser	Gly	Arg	Lys	Val	Asn	Cys	Thr	Thr	Gln	Pro	Cys
	1520					1525					1530			
Pro	Thr	Ala	Lys	Ala	Pro	Thr	Cys	Gly	Leu	Cys	Glu	Val	Ala	Arg
	1535					1540					1545			

Leu Arg Gln Asn Ala Asp Gln Cys Cys Pro Glu Tyr Glu Cys Val
1550 1555 1560

Cys Asp Pro Val Ser Cys Asp Leu Pro Pro Val Pro His Cys Glu
1565 1570 1575

Arg Gly Leu Gln Pro Thr Leu Thr Asn Pro Gly Glu Cys Arg Pro
1580 1585 1590

Asn Phe Thr Cys Ala Cys Arg Lys Glu Glu Cys Lys Arg Val Ser
1595 1600 1605

Pro Pro Ser Cys Pro Pro His Arg Leu Pro Thr Leu Arg Lys Thr
1610 1615 1620

Gln Cys Cys Asp Glu Tyr Glu Cys Ala Cys Asn Cys Val Asn Ser
1625 1630 1635

Thr Val Ser Cys Pro Leu Gly Tyr Leu Ala Ser Thr Ala Thr Asn
1640 1645 1650

Asp Cys Gly Cys Thr Thr Thr Thr Cys Leu Pro Asp Lys Val Cys
1655 1660 1665

Val His Arg Ser Thr Ile Tyr Pro Val Gly Gln Phe Trp Glu Glu
1670 1675 1680

Gly Cys Asp Val Cys Thr Cys Thr Asp Met Glu Asp Ala Val Met
1685 1690 1695

Gly Leu Arg Val Ala Gln Cys Ser Gln Lys Pro Cys Glu Asp Ser
1700 1705 1710

Cys Arg Ser Gly Phe Thr Tyr Val Leu His Glu Gly Glu Cys Cys
1715 1720 1725

Gly Arg Cys Leu Pro Ser Ala Cys Glu Val Val Thr Gly Ser Pro
1730 1735 1740

Arg Gly Asp Ser Gln Ser Ser Trp Lys Ser Val Gly Ser Gln Trp
1745 1750 1755

Ala Ser Pro Glu Asn Pro Cys Leu Ile Asn Glu Cys Val Arg Val
1760 1765 1770

Lys Glu Glu Val Phe Ile Gln Gln Arg Asn Val Ser Cys Pro Gln
1775 1780 1785

Leu Glu Val Pro Val Cys Pro Ser Gly Phe Gln Leu Ser Cys Lys
1790 1795 1800

Thr Ser Ala Cys Cys Pro Ser Cys Arg Cys Glu Arg Met Glu Ala
1805 1810 1815

Cys Met Leu Asn Gly Thr Val Ile Gly Pro Gly Lys Thr Val Met
1820 1825 1830

1820	1825	1830
Ile Asp Val Cys Thr Thr Cys Arg Cys Met Val Gln Val Gly Val 1835 1840 1845		
Ile Ser Gly Phe Lys Leu Glu Cys Arg Lys Thr Thr Cys Asn Pro 1850 1855 1860		
Cys Pro Leu Gly Tyr Lys Glu Glu Asn Asn Thr Gly Glu Cys Cys 1865 1870 1875		
Gly Arg Cys Leu Pro Thr Ala Cys Thr Ile Gln Leu Arg Gly Gly 1880 1885 1890		
Gln Ile Met Thr Leu Lys Arg Asp Glu Thr Leu Gln Asp Gly Cys 1895 1900 1905		
Asp Thr His Phe Cys Lys Val Asn Glu Arg Gly Glu Tyr Phe Trp 1910 1915 1920		
Glu Lys Arg Val Thr Gly Cys Pro Pro Phe Asp Glu His Lys Cys 1925 1930 1935		
Leu Ala Glu Gly Gly Lys Ile Met Lys Ile Pro Gly Thr Cys Cys 1940 1945 1950		
Asp Thr Cys Glu Glu Pro Glu Cys Asn Asp Ile Thr Ala Arg Leu 1955 1960 1965		
Gln Tyr Val Lys Val Gly Ser Cys Lys Ser Glu Val Glu Val Asp 1970 1975 1980		
Ile His Tyr Cys Gln Gly Lys Cys Ala Ser Lys Ala Met Tyr Ser 1985 1990 1995		
Ile Asp Ile Asn Asp Val Gln Asp Gln Cys Ser Cys Cys Ser Pro 2000 2005 2010		
Thr Arg Thr Glu Pro Met Gln Val Ala Leu His Cys Thr Asn Gly 2015 2020 2025		
Ser Val Val Tyr His Glu Val Leu Asn Ala Met Glu Cys Lys Cys 2030 2035 2040		
Ser Pro Arg Lys Cys Ser Lys 2045 2050		

<210> 5

<211> 479

<212> PRT

<213> Artificial sequence

<220>

<223> Modified D'-D3 domain of VWFPR

<400> 5

Pro Leu Trp Cys Arg Pro Pro Met Val Lys Leu Val Cys Pro Ala Asp
1 5 10 15

Asn Leu Arg Ala Glu Gly Leu Glu Cys Thr Lys Thr Cys Gln Asn Tyr
20 25 30

Asp Leu Glu Cys Met Ser Met Gly Cys Val Ser Gly Cys Leu Cys Pro
35 40 45

Pro Gly Met Val Arg His Glu Asn Arg Cys Val Ala Leu Glu Arg Cys
50 55 60

Pro Cys Phe His Gln Gly Lys Glu Tyr Ala Pro Gly Glu Thr Val Lys
65 70 75 80

Ile Gly Cys Asn Thr Cys Val Cys Arg Asp Arg Lys Trp Asn Cys Thr
85 90 95

Asp His Val Cys Asp Ala Thr Cys Ser Thr Ile Gly Met Ala His Tyr
100 105 110

Leu Thr Phe Asp Gly Leu Lys Tyr Leu Phe Pro Gly Glu Cys Gln Tyr
115 120 125

Val Leu Val Gln Asp Tyr Cys Gly Ser Asn Pro Gly Thr Phe Arg Ile
130 135 140

Leu Val Gly Asn Lys Gly Cys Ser His Pro Ser Val Lys Cys Lys Lys
145 150 155 160

Arg Val Thr Ile Leu Val Glu Gly Gly Glu Ile Glu Leu Phe Asp Gly
165 170 175

Glu Val Asn Val Lys Arg Pro Met Lys Asp Glu Thr His Phe Glu Val
180 185 190

Val Glu Ser Gly Arg Tyr Ile Ile Leu Leu Leu Gly Lys Ala Leu Ser
195 200 205

Val Val Trp Asp Arg His Leu Ser Ile Ser Val Val Leu Lys Gln Thr
210 215 220

Tyr Gln Glu Lys Val Cys Gly Leu Cys Gly Asn Phe Asp Gly Ile Gln
225 230 235 240

Asn Asn Asp Leu Thr Ser Ser Asn Leu Gln Val Glu Glu Asp Pro Val
245 250 255

Asp Phe Gly Asn Ser Trp Lys Val Ser Ser Gln Cys Ala Asp Thr Arg
260 265 270

Lys Val Pro Leu Asp Ser Ser Pro Ala Thr Cys His Asn Asn Ile Met
275 280 285

Lys Gln Thr Met Val Asp Ser Ser Cys Arg Ile Leu Thr Ser Asp Val
290 295 300

Phe Gln Asp Cys Asn Lys Leu Val Asp Pro Glu Pro Tyr Leu Asp Ala
305 310 315 320

Cys Ile Tyr Asp Thr Cys Ser Cys Glu Ser Ile Gly Asp Cys Ala Cys
325 330 335

Phe Cys Asp Thr Ile Ala Ala Tyr Ala His Val Cys Ala Gln His Gly
340 345 350

Lys Val Val Thr Trp Arg Thr Ala Thr Leu Cys Pro Gln Ser Cys Glu
355 360 365

Glu Arg Asn Leu Arg Glu Asn Gly Tyr Glu Cys Glu Trp Arg Tyr Asn
370 375 380

Ser Cys Ala Pro Ala Cys Gln Val Thr Cys Gln His Pro Glu Pro Leu
385 390 395 400

Ala Cys Pro Val Gln Cys Val Glu Gly Cys His Ala His Cys Pro Pro
405 410 415

Gly Lys Ile Leu Asp Glu Leu Leu Gln Thr Cys Val Asp Pro Glu Asp
420 425 430

Cys Pro Val Cys Glu Val Ala Gly Arg Arg Phe Ala Ser Gly Lys Lys
435 440 445

Val Thr Leu Asn Pro Ser Asp Pro Glu His Cys Gln Ile Cys His Cys
450 455 460

Asp Val Val Asn Leu Thr Cys Glu Ala Cys Gln Glu Pro Gly Gly
465 470 475

<210> 6

<211> 479

<212> PRT

<213> Artificial sequence

<220>

<223> Modified D'-D3 domain of VWFPRT

<400> 6

Gly Leu Tyr Cys Arg Pro Pro Met Val Lys Leu Val Cys Pro Ala Asp
1 5 10 15

Asn Leu Arg Ala Glu Gly Leu Glu Cys Thr Lys Thr Cys Gln Asn Tyr
20 25 30

Asp Leu Glu Cys Met Ser Met Gly Cys Val Ser Gly Cys Leu Cys Pro
35 40 45

Pro Glu Met Val Asp His Glu Asp Asp Cys Val Ala Leu Glu Asp Cys

Pro Gly Met Val Arg His Glu Asn Arg Cys Val Ala Leu Glu Arg Cys
 50 55 60

Pro Cys Phe His Gln Gly Lys Glu Tyr Ala Pro Gly Glu Thr Val Lys
 65 70 75 80

Ile Gly Cys Asn Thr Cys Val Cys Arg Asp Arg Lys Trp Asn Cys Thr
 85 90 95

Asp His Val Cys Asp Ala Thr Cys Ser Thr Ile Gly Met Ala His Tyr
 100 105 110

Leu Thr Phe Asp Gly Leu Lys Tyr Leu Phe Pro Gly Glu Cys Gln Tyr
 115 120 125

Val Leu Val Gln Asp Tyr Cys Gly Ser Asn Pro Gly Thr Phe Arg Ile
 130 135 140

Leu Val Gly Asn Lys Gly Cys Ser His Pro Ser Val Lys Cys Lys Lys
 145 150 155 160

Arg Val Thr Ile Leu Val Glu Gly Gly Glu Ile Glu Leu Phe Asp Gly
 165 170 175

Glu Val Asn Val Lys Arg Pro Met Lys Asp Glu Thr His Phe Glu Val
 180 185 190

Val Glu Ser Gly Arg Tyr Ile Ile Leu Leu Leu Gly Lys Ala Leu Ser
 195 200 205

Val Val Trp Asp Arg His Leu Ser Ile Ser Val Val Leu Lys Gln Thr
 210 215 220

Tyr Gln Glu Lys Val Cys Gly Leu Cys Gly Asn Phe Asp Gly Ile Gln
 225 230 235 240

Asn Asn Asp Leu Thr Ser Ser Asn Leu Gln Val Glu Glu Asp Pro Val
 245 250 255

Asp Phe Gly Asn Ser Trp Lys Val Ser Ser Gln Cys Ala Asp Thr Arg
 260 265 270

Lys Val Pro Leu Asp Ser Ser Pro Ala Thr Cys His Asn Asn Ile Met
 275 280 285

Lys Gln Thr Met Val Asp Ser Ser Cys Arg Ile Leu Thr Ser Asp Val
 290 295 300

Phe Gln Asp Cys Asn Lys Leu Val Asp Pro Glu Pro Tyr Leu Asp Ala
 305 310 315 320

Cys Ile Tyr Asp Thr Cys Ser Cys Glu Ser Ile Gly Asp Cys Ala Cys
 325 330 335

Phe Cys Asp Thr Ile Ala Ala Tyr Ala His Val Cys Ala Gln His Gly

340 345 350
 Lys Val Val Thr Trp Arg Thr Ala Thr Leu Cys Pro Gln Ser Cys Glu
 355 360 365
 Glu Arg Asn Leu Arg Glu Asn Gly Tyr Glu Cys Glu Trp Arg Tyr Asn
 370 375 380
 Ser Cys Ala Pro Ala Cys Gln Val Thr Cys Gln His Pro Glu Pro Leu
 385 390 395 400
 Ala Cys Pro Val Gln Cys Val Glu Gly Cys His Ala His Cys Pro Pro
 405 410 415
 Gly Lys Ile Leu Asp Glu Leu Leu Gln Thr Cys Val Asp Pro Glu Asp
 420 425 430
 Cys Pro Val Cys Glu Val Ala Gly Arg Arg Phe Ala Ser Gly Lys Lys
 435 440 445
 Val Thr Leu Asn Pro Ser Asp Pro Glu His Cys Gln Ile Cys His Cys
 450 455 460
 Asp Val Val Asn Leu Thr Cys Glu Ala Cys Gln Glu Pro Gly Gly
 465 470 475
 <210> 7
 <211> 479
 <212> PRT
 <213> Artificial sequence
 <220>
 <223> Modified D'-D3 domain of VWFPRT
 <400> 7
 Glu Leu Tyr Cys Arg Pro Pro Met Val Lys Leu Val Cys Pro Ala Asp
 1 5 10 15
 Asn Leu Arg Ala Glu Gly Leu Glu Cys Thr Lys Thr Cys Gln Asn Tyr
 20 25 30
 Asp Leu Glu Cys Met Ser Met Gly Cys Val Ser Gly Cys Leu Cys Pro
 35 40 45
 Pro Gly Met Val Arg His Glu Asn Arg Cys Val Ala Leu Glu Arg Cys
 50 55 60
 Pro Cys Phe His Gln Gly Lys Glu Tyr Ala Pro Gly Glu Thr Val Lys
 65 70 75 80
 Ile Gly Cys Asn Thr Cys Val Cys Arg Asp Arg Lys Trp Asn Cys Thr
 85 90 95
 Asp His Val Cys Asp Ala Thr Cys Ser Thr Ile Gly Met Ala His Tyr
 100 105 110

Leu Thr Phe Asp Gly Leu Lys Tyr Leu Phe Pro Gly Glu Cys Gln Tyr
 115 120 125

Val Leu Val Gln Asp Tyr Cys Gly Ser Asn Pro Gly Thr Phe Arg Ile
 130 135 140

Leu Val Gly Asn Lys Gly Cys Ser His Pro Ser Val Lys Cys Lys Lys
 145 150 155 160

Arg Val Thr Ile Leu Val Glu Gly Gly Glu Ile Glu Leu Phe Asp Gly
 165 170 175

Glu Val Asn Val Lys Arg Pro Met Lys Asp Glu Thr His Phe Glu Val
 180 185 190

Val Glu Ser Gly Arg Tyr Ile Ile Leu Leu Leu Gly Lys Ala Leu Ser
 195 200 205

Val Val Trp Asp Arg His Leu Ser Ile Ser Val Val Leu Lys Gln Thr
 210 215 220

Tyr Gln Glu Lys Val Cys Gly Leu Cys Gly Asn Phe Asp Gly Ile Gln
 225 230 235 240

Asn Asn Asp Leu Thr Ser Ser Asn Leu Gln Val Glu Glu Asp Pro Val
 245 250 255

Asp Phe Gly Asn Ser Trp Lys Val Ser Ser Gln Cys Ala Asp Thr Arg
 260 265 270

Lys Val Pro Leu Asp Ser Ser Pro Ala Thr Cys His Asn Asn Ile Met
 275 280 285

Lys Gln Thr Met Val Asp Ser Ser Cys Arg Ile Leu Thr Ser Asp Val
 290 295 300

Phe Gln Asp Cys Asn Lys Leu Val Asp Pro Glu Pro Tyr Leu Asp Ala
 305 310 315 320

Cys Ile Tyr Asp Thr Cys Ser Cys Glu Ser Ile Gly Asp Cys Ala Cys
 325 330 335

Phe Cys Asp Thr Ile Ala Ala Tyr Ala His Val Cys Ala Gln His Gly
 340 345 350

Lys Val Val Thr Trp Arg Thr Ala Thr Leu Cys Pro Gln Ser Cys Glu
 355 360 365

Glu Arg Asn Leu Arg Glu Asn Gly Tyr Glu Cys Glu Trp Arg Tyr Asn
 370 375 380

Ser Cys Ala Pro Ala Cys Gln Val Thr Cys Gln His Pro Glu Pro Leu
 385 390 395 400

Ala Cys Pro Val Gln Cys Val Glu Gly Cys His Ala His Cys Pro Pro
405 410 415

Gly Lys Ile Leu Asp Glu Leu Leu Gln Thr Cys Val Asp Pro Glu Asp
420 425 430

Cys Pro Val Cys Glu Val Ala Gly Arg Arg Phe Ala Ser Gly Lys Lys
435 440 445

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Val Thr Leu Asn Pro Ser Asp Pro Glu His Cys Gln Ile Cys His Cys
      450                      455                      460

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Asp Val Val Asn Leu Thr Cys Glu Ala Cys Gln Glu Pro Gly Gly
465 470 475

$\langle 210 \rangle$ 8

<211> 479

<212> PRT

<213> Artificial sequence

$\langle 220 \rangle$

<223> Modified D'-D3 domain of VWFPRT

$\langle 400 \rangle$ 8

Ser Leu Ser Cys Arg Pro Pro Met Val Lys Leu Val Cys Pro Ala Asp
1 5 10 15

Asn Leu Arg Ala Glu Gly Leu Glu Cys Thr Lys Thr Cys Gln Asn Tyr
20 25 30

Asp Leu Glu Cys Met Ser Met Gly Cys Val Ser Gly Cys Leu Cys Pro
35 40 45

Pro Gly Met Val Arg His Glu Asn Arg Cys Val Ala Leu Glu Arg Cys
50 55 60

Pro Cys Phe His Gln Gly Lys Glu Tyr Ala Pro Gly Glu Thr Val Lys
65 70 75 80

Ile Gly Cys Asn Thr Cys Val Cys Arg Asp Arg Lys Trp Asn Cys Thr
85 90 95

Asp His Val Cys Asp Ala Thr Cys Ser Thr Ile Gly Met Ala His Tyr
100 105 110

Leu Thr Phe Asp Gly Leu Lys Tyr Leu Phe Pro Gly Glu Cys Gln Tyr

115 120 125

Val	Leu	Val	Gln	Asp	Tyr	Cys	Gly	Ser	Asn	Pro	Gly	Thr	Phe	Arg	Ile
130						135					140				

Leu Val Gly Asn Lys Gly Cys Ser His Pro Ser Val Lys Cys Lys Lys
145 150 155 160

Arg Val Thr Ile Leu Val Glu Gly Gly Glu Ile Glu Leu Phe Asp Gly
 165 170 175

Glu Val Asn Val Lys Arg Pro Met Lys Asp Glu Thr His Phe Glu Val
 180 185 190

Val Glu Ser Gly Arg Tyr Ile Ile Leu Leu Leu Gly Lys Ala Leu Ser
 195 200 205

Val Val Trp Asp Arg His Leu Ser Ile Ser Val Val Leu Lys Gln Thr
 210 215 220

Tyr Gln Glu Lys Val Cys Gly Leu Cys Gly Asn Phe Asp Gly Ile Gln
 225 230 235 240

Asn Asn Asp Leu Thr Ser Ser Ser Leu Gln Val Glu Glu Asp Pro Val
 245 250 255

Asp Phe Gly Asn Ser Trp Lys Val Ser Ser Gln Cys Ala Asp Thr Arg
 260 265 270

Lys Val Pro Leu Asp Ser Ser Pro Ala Thr Cys His Asn Asn Ile Met
 275 280 285

Lys Gln Thr Met Val Asp Ser Ser Cys Arg Ile Leu Thr Ser Asp Val
 290 295 300

Phe Gln Asp Cys Asn Lys Leu Val Asp Pro Glu Pro Tyr Leu Asp Ala
 305 310 315 320

Cys Ile Tyr Asp Thr Cys Ser Cys Glu Ser Ile Gly Asp Cys Ala Cys
 325 330 335

Phe Cys Asp Thr Ile Ala Ala Tyr Ala His Val Cys Ala Gln His Gly
 340 345 350

Lys Val Val Thr Trp Arg Thr Ala Thr Leu Cys Pro Gln Ser Cys Glu
 355 360 365

Glu Arg Asn Leu Arg Glu Asn Gly Tyr Glu Cys Glu Trp Arg Tyr Asn
 370 375 380

Ser Cys Ala Pro Ala Cys Gln Val Thr Cys Gln His Pro Glu Pro Leu
 385 390 395 400

Ala Cys Pro Val Gln Cys Val Glu Gly Cys His Ala His Cys Pro Pro
 405 410 415

Gly Glu Ile Leu Asp Glu Leu Leu Gln Thr Cys Val Asp Pro Glu Asp
 420 425 430

Cys Pro Val Cys Glu Val Ala Gly Arg Arg Phe Ala Ser Gly Lys Lys
 435 440 445

Val Thr Leu Asn Pro Ser Asp Pro Glu His Cys Gln Ile Cys His Cys
 450 455 460

Asp Val Val Asn Leu Thr Cys Glu Ala Cys Gln Glu Pro Gly Gly
 465 470 475

<210> 9

<211> 479

<212> PRT

<213> Artificial sequence

<220>

<223> Modified D'-D3 domain of VWFPR

<400> 9

Ser Leu Ser Cys Arg Pro Pro Met Val Lys Leu Val Cys Pro Ala Asp
 1 5 10 15

Asn Leu Arg Ala Glu Gly Leu Glu Cys Thr Lys Thr Cys Gln Asn Tyr
 20 25 30

Asp Leu Glu Cys Met Ser Met Gly Cys Val Ser Gly Cys Leu Cys Pro
 35 40 45

Pro Gly Met Val Arg His Glu Asn Arg Cys Val Ala Leu Glu Arg Cys
 50 55 60

Pro Cys Phe His Gln Gly Lys Glu Tyr Ala Pro Gly Glu Thr Val Lys
 65 70 75 80

Ile Gly Cys Asn Thr Cys Val Cys Arg Asp Arg Lys Trp Asn Cys Thr
 85 90 95

Asp His Val Cys Asp Ala Thr Cys Ser Thr Ile Gly Met Ala His Tyr

100

105

110

Leu Thr Phe Asp Gly Leu Lys Tyr Leu Phe Pro Gly Glu Cys Gln Tyr
 115 120 125

Val Leu Val Gln Asp Tyr Cys Gly Ser Asn Pro Gly Thr Phe Arg Ile
 130 135 140

Leu Val Gly Asn Lys Gly Cys Ser His Pro Ser Val Lys Cys Lys Lys
 145 150 155 160

Arg Val Thr Ile Leu Val Glu Gly Gly Glu Ile Glu Leu Phe Asp Gly
 165 170 175

Glu Val Asn Val Lys Arg Pro Met Lys Asp Glu Thr His Phe Glu Val
 180 185 190

Val Glu Ser Gly Arg Tyr Ile Ile Leu Leu Leu Gly Lys Ala Leu Ser
 195 200 205

Val Val Trp Asp Arg His Leu Ser Ile Ser Val Val Leu Lys Gln Thr

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210      -      -      -      215      220      -
Tyr Gln Glu Lys Val Cys Gly Leu Cys Gly Asn Phe Asp Gly Ile Gln
225      230      235      240
Asn Asn Asp Leu Thr Ser Ser Asn Leu Gln Val Glu Glu Asp Pro Val
245      250      255
Asp Phe Gly Asn Ser Trp Lys Val Ser Ser Gln Cys Ala Asp Thr Arg
260      265      270
Lys Val Pro Leu Asp Ser Ser Pro Ala Thr Cys His Asn Asn Ile Met
275      280      285
Lys Gln Thr Met Val Asp Ser Ser Cys Arg Ile Leu Thr Ser Asp Val
290      295      300
Phe Gln Asp Cys Asn Lys Leu Val Asp Pro Glu Pro Tyr Leu Asp Ala
305      310      315      320
Cys Ile Tyr Asp Thr Cys Ser Cys Glu Ser Ile Gly Asp Cys Ala Cys
325      330      335
Phe Cys Asp Thr Ile Ala Ala Tyr Ala His Val Cys Ala Gln His Gly
340      345      350
Lys Val Val Thr Trp Arg Thr Ala Thr Leu Cys Pro Gln Ser Cys Glu
355      360      365
Glu Arg Asn Leu Arg Glu Asn Gly Tyr Glu Cys Glu Trp Arg Tyr Asn
370      375      380
Ser Cys Ala Pro Ala Cys Gln Val Thr Cys Gln His Pro Glu Pro Leu
385      390      395      400
Ala Cys Pro Val Gln Cys Val Glu Gly Cys His Ala His Cys Pro Pro
405      410      415
Gly Lys Ile Leu Asp Glu Leu Leu Gln Thr Cys Val Asp Pro Glu Asp
420      425      430
Cys Pro Val Cys Glu Val Ala Gly Arg Arg Phe Ala Ser Gly Lys Lys
435      440      445
Val Thr Leu Asn Pro Ser Asp Pro Glu His Cys Gln Ile Cys His Cys
450      455      460
Asp Val Val Asn Leu Thr Cys Glu Ala Cys Gln Glu Pro Gly Gly
465      470      475

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<210> 10

<211> 479

<212> PRT

<213> Artificial sequence

<220>

<223> Modified D'-D3 domain of VWFPR

<400> 10

Ser Leu Ser Cys Arg Pro Pro Met Val Lys Leu Val Cys Pro Ala Asp
 1 5 10 15

Asn Leu Arg Ala Glu Gly Leu Glu Cys Thr Lys Thr Cys Gln Asn Tyr
 20 25 30

Asp Leu Glu Cys Met Ser Met Gly Cys Val Ser Gly Cys Leu Cys Pro
 35 40 45

Pro Gly Met Val Arg His Glu Asn Arg Cys Val Ala Leu Glu Arg Cys
 50 55 60

Pro Cys Phe His Gln Gly Lys Glu Tyr Ala Pro Gly Glu Thr Val Lys
 65 70 75 80

Ile Gly Cys Asn Thr Cys Val Cys Arg Asp Arg Lys Trp Asn Cys Thr
 85 90 95

Asp His Val Cys Asp Ala Thr Cys Ser Thr Ile Gly Met Ala His Tyr
 100 105 110

Leu Thr Phe Asp Gly Leu Lys Tyr Leu Phe Pro Gly Glu Cys Gln Tyr
 115 120 125

Val Leu Val Gln Asp Tyr Cys Gly Ser Asn Pro Gly Thr Phe Arg Ile
 130 135 140

Leu Val Gly Asn Lys Gly Cys Ser His Pro Ser Val Lys Cys Lys Lys
 145 150 155 160

Arg Val Thr Ile Leu Val Glu Gly Gly Glu Ile Glu Leu Phe Asp Gly
 165 170 175

Glu Val Asn Val Lys Arg Pro Met Lys Asp Glu Thr His Phe Glu Val
 180 185 190

Val Glu Ser Gly Arg Tyr Ile Ile Leu Leu Leu Gly Lys Ala Leu Ser
 195 200 205

Val Val Trp Asp Arg His Leu Ser Ile Ser Val Val Leu Lys Gln Thr
 210 215 220

Tyr Gln Glu Lys Val Cys Gly Leu Cys Gly Asn Phe Asp Gly Ile Gln
 225 230 235 240

Asn Asn Asp Leu Thr Ser Ser Asn Leu Gln Val Glu Glu Asp Pro Val
 245 250 255

Asp Phe Gly Asn Ser Trp Lys Val Ser Ser Gln Cys Ala Asp Thr Arg
 260 265 270

Lys Val Pro Leu Asp Ser Thr Pro Ala Thr Cys His Asn Asn Ile Met
 275 280 285

Lys Gln Thr Met Val Asp Ser Ser Cys Arg Ile Leu Thr Ser Asp Val
 290 295 300

Phe Gln Asp Cys Asn Lys Leu Val Asp Pro Glu Pro Tyr Leu Asp Val
 305 310 315 320

Cys Ile Tyr Asp Thr Cys Ser Cys Glu Ser Ile Gly Asp Cys Ala Cys
 325 330 335

Phe Cys Asp Thr Ile Ala Ala Tyr Ala His Val Cys Ala Gln His Gly
 340 345 350

Lys Val Val Thr Trp Arg Thr Ala Thr Leu Cys Pro Gln Ser Cys Glu
 355 360 365

Glu Arg Asn Leu Arg Glu Asn Gly Tyr Glu Cys Glu Trp Arg Tyr Asn
 370 375 380

Ser Cys Ala Pro Ala Cys Gln Val Thr Cys Gln His Pro Glu Pro Leu
 385 390 395 400

Ala Cys Pro Val Gln Cys Val Glu Gly Cys His Ala His Cys Pro Pro
 405 410 415

Gly Lys Ile Leu Asp Glu Leu Leu Gln Thr Cys Val Asp Pro Glu Asp
 420 425 430

Cys Pro Val Cys Glu Val Ala Gly Arg Arg Phe Ala Ser Gly Lys Lys
 435 440 445

Val Thr Leu Asn Pro Ser Asp Pro Glu His Cys Gln Ile Cys His Cys
 450 455 460

Asp Val Val Asn Leu Thr Cys Glu Ala Cys Gln Glu Pro Gly Gly
 465 470 475

<210> 11

<211> 479

<212> PRT

<213> Artificial sequence

<220>

<223> Modified D'-D3 domain of VWFPR

<400> 11

Ser Leu Ser Cys Arg Pro Pro Met Val Lys Leu Val Cys Pro Ala Asp
 1 5 10 15

Asn Leu Arg Ala Glu Gly Leu Glu Cys Thr Lys Thr Cys Gln Asn Tyr
 20 25 30

Asp Leu Glu Cys Met Ser Met Gly Cys Ala Ser Gly Cys Leu Cys Pro
 35 40 45

Pro Gly Met Val Arg His Glu Asn Arg Cys Val Ala Leu Glu Arg Cys
 50 55 60

Pro Cys Phe His Gln Gly Lys Glu Tyr Ala Pro Gly Glu Thr Val Lys
 65 70 75 80

Ile Gly Cys Asn Thr Cys Val Cys Arg Asp Arg Lys Trp Asn Cys Thr
 85 90 95

Asp His Val Cys Asp Ala Thr Cys Ser Thr Ile Gly Met Ala His Tyr
 100 105 110

Leu Thr Phe Asp Gly Leu Lys Tyr Leu Phe Pro Gly Glu Cys Gln Tyr
 115 120 125

Val Leu Val Gln Asp Tyr Cys Gly Ser Asn Pro Gly Thr Phe Arg Ile
 130 135 140

Leu Val Gly Asn Lys Gly Cys Ser His Pro Ser Val Lys Cys Lys Lys
 145 150 155 160

Arg Val Thr Ile Leu Val Glu Gly Gly Glu Ile Glu Leu Phe Asp Gly
 165 170 175

Glu Val Asn Val Lys Arg Pro Met Lys Asp Glu Thr His Phe Glu Val
 180 185 190

Val Glu Ser Gly Arg Tyr Ile Ile Leu Leu Leu Gly Lys Ala Leu Ser
 195 200 205

Val Val Trp Asp Arg His Leu Ser Ile Ser Val Val Leu Lys Gln Thr
 210 215 220

Tyr Gln Glu Lys Val Cys Gly Leu Cys Gly Asn Phe Asp Gly Ile Gln
 225 230 235 240

Asn Asn Asp Leu Thr Ser Ser Asn Leu Gln Val Glu Glu Asp Pro Val
 245 250 255

Asp Phe Gly Asn Ser Trp Lys Val Ser Ser Gln Cys Ala Asp Thr Arg
 260 265 270

Lys Val Pro Leu Asp Ser Ser Pro Ala Thr Cys His Asn Asn Ile Met
 275 280 285

Lys Gln Thr Met Val Asp Ser Ser Cys Arg Ile Leu Thr Ser Asp Val
 290 295 300

Phe Gln Asp Cys Asn Lys Leu Val Asp Pro Glu Pro Tyr Leu Asp Val
 305 310 315 320

Cys Ile Tyr Asp Thr Cys Ser Cys Glu Ser Ile Gly Asp Cys Ala Cys
 325 330 335

340 350 355
 Phe Cys Asp Thr Ile Ala Ala Tyr Ala His Val Cys Ala Gln His Gly
 340 345 350
 Lys Val Val Thr Trp Arg Thr Ala Thr Leu Cys Pro Gln Ser Cys Glu
 355 360 365
 Glu Arg Asn Leu Arg Glu Asn Gly Tyr Glu Cys Glu Trp Arg Tyr Asn
 370 375 380
 Ser Cys Ala Pro Ala Cys Gln Val Thr Cys Leu His Pro Glu Pro Leu
 385 390 395 400
 Ala Cys Pro Val Gln Cys Val Glu Gly Cys His Ala His Cys Pro Pro
 405 410 415
 Gly Lys Ile Leu Asp Glu Leu Leu Gln Thr Cys Val Asp Pro Glu Asp
 420 425 430
 Cys Pro Val Cys Glu Val Ala Gly Arg Arg Phe Ala Ser Gly Lys Lys
 435 440 445
 Val Thr Leu Asn Pro Ser Asp Pro Glu His Cys Gln Ile Cys His Cys
 450 455 460
 Asp Val Val Asn Leu Thr Cys Glu Ala Cys Gln Glu Pro Gly Gly
 465 470 475

<210> 12

<211> 479

<212> PRT

<213> Artificial sequence

<220>

<223> Modified D'-D3 domain of VWFPR

<400> 12

Ser Leu Ser Cys Arg Pro Pro Met Val Lys Leu Val Cys Pro Ala Asp
 1 5 10 15
 Asn Leu Arg Ala Glu Gly Leu Glu Cys Thr Lys Thr Cys Gln Asn Tyr
 20 25 30
 Asp Leu Glu Cys Met Ser Met Gly Cys Val Ser Gly Cys Leu Cys Pro
 35 40 45
 Pro Gly Met Val Arg His Glu Asn Arg Cys Val Ala Leu Glu Arg Cys
 50 55 60
 Pro Cys Phe His Gln Gly Lys Glu Tyr Ala Pro Gly Glu Thr Val Lys
 65 70 75 80
 Ile Glu Cys Asp Thr Cys Val Cys Arg Asp Arg Lys Thr Asp Cys Thr

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Ile Gly Cys Asn Thr Cys Val Cys Arg Asp Arg Lys Trp Asn Cys Thr
      85                                90                                95

Asp His Val Cys Asp Ala Thr Cys Ser Thr Ile Gly Met Ala His Tyr
      100                                105                                110

Leu Thr Phe Asp Gly Leu Lys Tyr Leu Phe Pro Gly Glu Cys Gln Tyr
      115                                120                                125

Val Leu Val Gln Asp Tyr Cys Gly Ser Asn Pro Gly Thr Phe Arg Ile
      130                                135                                140

Leu Val Gly Asn Glu Gly Cys Ser His Pro Ser Val Lys Cys Lys Lys
      145                                150                                155                                160

Arg Val Thr Ile Leu Val Glu Gly Gly Glu Ile Glu Leu Phe Asp Gly
      165                                170                                175

Glu Val Asn Val Lys Arg Pro Met Lys Asp Glu Thr His Phe Glu Val
      180                                185                                190

Val Glu Ser Gly Arg Tyr Ile Ile Leu Leu Leu Gly Lys Ala Leu Ser
      195                                200                                205

Val Val Trp Asp Arg His Leu Ser Ile Ser Val Val Leu Lys Gln Thr
      210                                215                                220

Tyr Gln Glu Lys Val Cys Gly Leu Cys Gly Asn Phe Asp Gly Ile Gln
      225                                230                                235                                240

Asn Asn Asp Leu Thr Ser Ser Asn Leu Gln Val Glu Glu Asp Pro Val
      245                                250                                255

Asp Phe Gly Asn Ser Trp Lys Val Ser Ser Gln Cys Ala Asp Thr Arg
      260                                265                                270

Lys Val Pro Leu Asp Ser Ser Pro Ala Thr Cys His Asn Asn Ile Met
      275                                280                                285

Lys Gln Thr Met Val Asp Ser Ser Cys Arg Ile Leu Thr Ser Asp Val
      290                                295                                300

Phe Gln Asp Cys Asn Lys Leu Val Asp Pro Glu Pro Tyr Leu Asp Val
      305                                310                                315                                320

Cys Ile Tyr Asp Ser Cys Ser Cys Glu Ser Ile Gly Asp Cys Ala Cys
      325                                330                                335

Phe Cys Asp Thr Ile Ala Ala Tyr Ala His Val Cys Ala Gln His Gly
      340                                345                                350

Lys Val Val Thr Trp Arg Thr Ala Thr Leu Cys Pro Gln Ser Cys Glu
      355                                360                                365

Glu Arg Asn Leu Arg Glu Asn Gly Tyr Glu Cys Glu Trp Arg Tyr Asn
      370                                375                                380

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Ser Cys Ala Pro Ala Cys Gln Val Thr Cys Gln His Pro Glu Pro Leu
385 390 395 400

Ala Cys Pro Val Gln Cys Val Glu Gly Cys His Ala His Cys Pro Pro
405 410 415

Gly Lys Ile Leu Asp Glu Leu Leu Gln Thr Cys Val Asp Pro Glu Asp
420 425 430

Cys Pro Val Cys Glu Val Ala Gly Arg Arg Phe Ala Ser Gly Lys Lys
435 440 445

Val Thr Leu Asn Pro Ser Asp Pro Glu His Cys Gln Ile Cys His Cys
450 455 460

Asp Val Val Asn Leu Thr Cys Glu Ala Cys Gln Glu Pro Gly Gly
465 470 475

<210> 13

<211> 479

<212> PRT

<213> Artificial sequence

<220>

<223> Modified D'-D3 domain of VWFPR

<400> 13

Ser Leu Ser Cys Arg Pro Pro Met Val Lys Leu Val Cys Pro Ala Asp
1 5 10 15

Asn Pro Arg Ala Glu Gly Leu Glu Cys Thr Lys Thr Cys Gln Asn Tyr
20 25 30

Asp Leu Glu Cys Met Ser Met Gly Cys Val Ser Gly Cys Leu Cys Pro
35 40 45

Pro Gly Met Val Arg His Glu Asn Arg Cys Val Ala Leu Glu Arg Cys
50 55 60

Pro Cys Phe His Gln Gly Lys Glu Tyr Ala Pro Gly Glu Thr Val Lys
65 70 75 80

Ile Gly Cys Asn Thr Cys Val Cys Arg Asp Arg Lys Trp Asn Cys Thr
85 90 95

Asp His Val Cys Asp Ala Thr Cys Ser Thr Ile Gly Met Ala His Tyr
100 105 110

Leu Thr Phe Asp Gly Leu Lys Tyr Leu Phe Pro Gly Glu Cys Gln Tyr
115 120 125

Val Leu Val Gln Asp Tyr Cys Gly Ser Asn Pro Gly Thr Phe Arg Ile
130 135 140

Leu Val Gly Asn Lys Gly Cys Ser His Pro Ser Val Lys Cys Lys Lys
 145 150 155 160

Arg Val Thr Ile Leu Val Glu Gly Gly Glu Ile Glu Leu Phe Asp Gly
 165 170 175

Glu Val Asn Val Lys Arg Pro Met Lys Asp Glu Thr His Phe Glu Val
 180 185 190

Val Glu Ser Gly Arg Tyr Ile Ile Leu Leu Leu Gly Lys Ala Leu Ser
 195 200 205

Val Val Trp Asp Arg His Leu Ser Ile Ser Val Val Leu Lys Gln Thr
 210 215 220

Tyr Gln Glu Lys Val Cys Gly Leu Cys Gly Asn Phe Asp Gly Ile Gln
 225 230 235 240

Asn Asn Asp Leu Thr Ser Ser Asn Leu Gln Val Glu Glu Asp Pro Val
 245 250 255

Asp Phe Gly Asn Ser Trp Lys Val Ser Ser Gln Cys Ala Asp Thr Arg
 260 265 270

Lys Val Pro Leu Asp Ser Ser Pro Ala Thr Cys His Asn Asn Ile Met
 275 280 285

Lys Gln Thr Met Val Asp Ser Ser Cys Arg Ile Leu Thr Ser Asp Val
 290 295 300

Phe Gln Asp Cys Asn Lys Leu Val Asp Pro Glu Pro Tyr Leu Asp Val
 305 310 315 320

Cys Ile Tyr Asp Thr Cys Ser Cys Glu Ser Ile Gly Asp Cys Ala Cys
 325 330 335

Phe Cys Asp Thr Ile Ala Ala Tyr Ala His Val Cys Ala Gln His Gly
 340 345 350

Lys Val Val Thr Trp Arg Thr Ala Thr Leu Cys Pro Gln Ser Cys Glu
 355 360 365

Glu Arg Asn Leu Arg Glu Asn Gly Tyr Glu Cys Glu Trp Arg Tyr Asn
 370 375 380

Ser Cys Ala Pro Ala Cys Gln Val Thr Cys Gln His Pro Glu Pro Leu
 385 390 395 400

Ala Cys Pro Val Gln Cys Val Glu Gly Cys His Ala His Cys Pro Pro
 405 410 415

Gly Lys Ile Leu Asp Glu Leu Leu Gln Thr Cys Val Asp Pro Glu Asp
 420 425 430

Cys Pro Val Cys Glu Val Ala Gly Arg Arg Phe Ala Ser Gly Lys Lys
 435 440 445

430 440 450
 Val Thr Leu Asn Pro Ser Asp Pro Glu His Cys Gln Ile Cys His Cys
 450 455 460

 Asp Val Val Asn Leu Thr Cys Glu Ala Cys Gln Glu Pro Gly Gly
 465 470 475

 <210> 14
 <211> 2332
 <212> PRT
 <213> Homo sapiens

 <400> 14
 Ala Thr Arg Arg Tyr Tyr Leu Gly Ala Val Glu Leu Ser Trp Asp Tyr
 1 5 10 15

 Met Gln Ser Asp Leu Gly Glu Leu Pro Val Asp Ala Arg Phe Pro Pro
 20 25 30

 Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val Tyr Lys Lys
 35 40 45

 Thr Leu Phe Val Glu Phe Thr Asp His Leu Phe Asn Ile Ala Lys Pro
 50 55 60

 Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln Ala Glu Val
 65 70 75 80

 Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser His Pro Val
 85 90 95

 Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser Glu Gly Ala
 100 105 110

 Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys Glu Asp Asp Lys Val
 115 120 125

 Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu Lys Glu Asn
 130 135 140

 Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser Tyr Leu Ser
 145 150 155 160

 His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile Gly Ala Leu
 165 170 175

 Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr Gln Thr Leu
 180 185 190

 His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly Lys Ser Trp
 195 200 205

 His Ser Glu Thr Lys Asn Ser Leu Met Gln Asp Arg Asp Ala Ala Ser
 210 215 220

 Ala Arg Ala Trp Pro Lys Met His Thr Val Asn Gly Tyr Val Asn Arg

225																	
Ser	Leu	Pro	Gly	Leu	Ile	Gly	Cys	His	Arg	Lys	Ser	Val	Tyr	Trp	His		
				245					250					255			
Val	Ile	Gly	Met	Gly	Thr	Thr	Pro	Glu	Val	His	Ser	Ile	Phe	Leu	Glu		
			260					265					270				
Gly	His	Thr	Phe	Leu	Val	Arg	Asn	His	Arg	Gln	Ala	Ser	Leu	Glu	Ile		
		275					280					285					
Ser	Pro	Ile	Thr	Phe	Leu	Thr	Ala	Gln	Thr	Leu	Leu	Met	Asp	Leu	Gly		
	290					295					300						
Gln	Phe	Leu	Leu	Phe	Cys	His	Ile	Ser	Ser	His	Gln	His	Asp	Gly	Met		
305					310					315					320		
Glu	Ala	Tyr	Val	Lys	Val	Asp	Ser	Cys	Pro	Glu	Glu	Pro	Gln	Leu	Arg		
				325					330					335			
Met	Lys	Asn	Asn	Glu	Glu	Ala	Glu	Asp	Tyr	Asp	Asp	Asp	Leu	Thr	Asp		
			340					345					350				
Ser	Glu	Met	Asp	Val	Val	Arg	Phe	Asp	Asp	Asp	Asn	Ser	Pro	Ser	Phe		
		355					360					365					
Ile	Gln	Ile	Arg	Ser	Val	Ala	Lys	Lys	His	Pro	Lys	Thr	Trp	Val	His		
	370					375					380						
Tyr	Ile	Ala	Ala	Glu	Glu	Glu	Asp	Trp	Asp	Tyr	Ala	Pro	Leu	Val	Leu		
385					390					395					400		
Ala	Pro	Asp	Asp	Arg	Ser	Tyr	Lys	Ser	Gln	Tyr	Leu	Asn	Asn	Gly	Pro		
				405					410					415			
Gln	Arg	Ile	Gly	Arg	Lys	Tyr	Lys	Lys	Val	Arg	Phe	Met	Ala	Tyr	Thr		
			420					425					430				
Asp	Glu	Thr	Phe	Lys	Thr	Arg	Glu	Ala	Ile	Gln	His	Glu	Ser	Gly	Ile		
		435					440					445					
Leu	Gly	Pro	Leu	Leu	Tyr	Gly	Glu	Val	Gly	Asp	Thr	Leu	Leu	Ile	Ile		
	450					455					460						
Phe	Lys	Asn	Gln	Ala	Ser	Arg	Pro	Tyr	Asn	Ile	Tyr	Pro	His	Gly	Ile		
465					470					475					480		
Thr	Asp	Val	Arg	Pro	Leu	Tyr	Ser	Arg	Arg	Leu	Pro	Lys	Gly	Val	Lys		
				485					490					495			
His	Leu	Lys	Asp	Phe	Pro	Ile	Leu	Pro	Gly	Glu	Ile	Phe	Lys	Tyr	Lys		
			500					505					510				
Trp	Thr	Val	Thr	Val	Glu	Asp	Gly	Pro	Thr	Lys	Ser	Asp	Pro	Arg	Cys		
		515					520						525				
Leu	Thr	Arg	Tyr	Tyr	Ser	Ser	Phe	Val	Asn	Met	Glu	Arg	Asp	Leu	Ala		

530	535	540
Ser Gly Leu Ile Gly	Pro Leu Leu Ile Cys Tyr Lys Glu Ser Val Asp	
545	550	555 560
Gln Arg Gly Asn Gln Ile Met Ser Asp Lys Arg Asn Val Ile Leu Phe		
	565	570 575
Ser Val Phe Asp Glu Asn Arg Ser Trp Tyr Leu Thr Glu Asn Ile Gln		
	580	585 590
Arg Phe Leu Pro Asn Pro Ala Gly Val Gln Leu Glu Asp Pro Glu Phe		
	595	600 605
Gln Ala Ser Asn Ile Met His Ser Ile Asn Gly Tyr Val Phe Asp Ser		
	610	615 620
Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp Tyr Ile Leu		
	625	630 635 640
Ser Ile Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe Ser Gly Tyr		
	645	650 655
Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr Leu Phe Pro		
	660	665 670
Phe Ser Gly Glu Thr Val Phe Met Ser Met Glu Asn Pro Gly Leu Trp		
	675	680 685
Ile Leu Gly Cys His Asn Ser Asp Phe Arg Asn Arg Gly Met Thr Ala		
	690	695 700
Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr Gly Asp Tyr Tyr Glu		
	705	710 715 720
Asp Ser Tyr Glu Asp Ile Ser Ala Tyr Leu Leu Ser Lys Asn Asn Ala		
	725	730 735
Ile Glu Pro Arg Ser Phe Ser Gln Asn Ser Arg His Arg Ser Thr Arg		
	740	745 750
Gln Lys Gln Phe Asn Ala Thr Thr Ile Pro Glu Asn Asp Ile Glu Lys		
	755	760 765
Thr Asp Pro Trp Phe Ala His Arg Thr Pro Met Pro Lys Ile Gln Asn		
	770	775 780
Val Ser Ser Ser Asp Leu Leu Met Leu Leu Arg Gln Ser Pro Thr Pro		
	785	790 795 800
His Gly Leu Ser Leu Ser Asp Leu Gln Glu Ala Lys Tyr Glu Thr Phe		
	805	810 815
Ser Asp Asp Pro Ser Pro Gly Ala Ile Asp Ser Asn Asn Ser Leu Ser		
	820	825 830

Glu Met Thr His Phe Arg Pro Gln Leu His His Ser Gly Asp Met Val
 835 840 845

Phe Thr Pro Glu Ser Gly Leu Gln Leu Arg Leu Asn Glu Lys Leu Gly
 850 855 860

Thr Thr Ala Ala Thr Glu Leu Lys Lys Leu Asp Phe Lys Val Ser Ser
 865 870 875 880

Thr Ser Asn Asn Leu Ile Ser Thr Ile Pro Ser Asp Asn Leu Ala Ala
 885 890 895

Gly Thr Asp Asn Thr Ser Ser Leu Gly Pro Pro Ser Met Pro Val His
 900 905 910

Tyr Asp Ser Gln Leu Asp Thr Thr Leu Phe Gly Lys Lys Ser Ser Pro
 915 920 925

Leu Thr Glu Ser Gly Gly Pro Leu Ser Leu Ser Glu Glu Asn Asn Asp
 930 935 940

Ser Lys Leu Leu Glu Ser Gly Leu Met Asn Ser Gln Glu Ser Ser Trp
 945 950 955 960

Gly Lys Asn Val Ser Ser Thr Glu Ser Gly Arg Leu Phe Lys Gly Lys
 965 970 975

Arg Ala His Gly Pro Ala Leu Leu Thr Lys Asp Asn Ala Leu Phe Lys
 980 985 990

Val Ser Ile Ser Leu Leu Lys Thr Asn Lys Thr Ser Asn Asn Ser Ala
 995 1000 1005

Thr Asn Arg Lys Thr His Ile Asp Gly Pro Ser Leu Leu Ile Glu
 1010 1015 1020

Asn Ser Pro Ser Val Trp Gln Asn Ile Leu Glu Ser Asp Thr Glu
 1025 1030 1035

Phe Lys Lys Val Thr Pro Leu Ile His Asp Arg Met Leu Met Asp
 1040 1045 1050

Lys Asn Ala Thr Ala Leu Arg Leu Asn His Met Ser Asn Lys Thr
 1055 1060 1065

Thr Ser Ser Lys Asn Met Glu Met Val Gln Gln Lys Lys Glu Gly
 1070 1075 1080

Pro Ile Pro Pro Asp Ala Gln Asn Pro Asp Met Ser Phe Phe Lys
 1085 1090 1095

Met Leu Phe Leu Pro Glu Ser Ala Arg Trp Ile Gln Arg Thr His
 1100 1105 1110

Gly Lys Asn Ser Leu Asn Ser Gly Gln Gly Pro Ser Pro Lys Gln
 1115 1120 1125

Leu Val 1130	Ser Leu Gly Pro	Glu Lys Ser Val	Glu Gly	Gln Asn Phe
		1135	1140	
Leu Ser 1145	Glu Lys Asn Lys	Val Val Val Gly Lys	Gly Glu Phe Thr	
		1150	1155	
Lys Asp 1160	Val Gly Leu Lys	Glu Met Val Phe Pro	Ser Ser Arg Asn	
		1165	1170	
Leu Phe 1175	Leu Thr Asn Leu	Asp Asn Leu His Glu	Asn Asn Thr His	
		1180	1185	
Asn Gln 1190	Glu Lys Lys Ile	Gln Glu Glu Ile Glu	Lys Lys Glu Thr	
		1195	1200	
Leu Ile 1205	Gln Glu Asn Val	Val Leu Pro Gln Ile	His Thr Val Thr	
		1210	1215	
Gly Thr 1220	Lys Asn Phe Met	Lys Asn Leu Phe Leu	Leu Ser Thr Arg	
		1225	1230	
Gln Asn 1235	Val Glu Gly Ser	Tyr Asp Gly Ala Tyr	Ala Pro Val Leu	
		1240	1245	
Gln Asp 1250	Phe Arg Ser Leu	Asn Asp Ser Thr Asn	Arg Thr Lys Lys	
		1255	1260	
His Thr 1265	Ala His Phe Ser	Lys Lys Gly Glu Glu	Glu Asn Leu Glu	
		1270	1275	
Gly Leu 1280	Gly Asn Gln Thr	Lys Gln Ile Val Glu	Lys Tyr Ala Cys	
		1285	1290	
Thr Thr 1295	Arg Ile Ser Pro	Asn Thr Ser Gln Gln	Asn Phe Val Thr	
		1300	1305	
Gln Arg 1310	Ser Lys Arg Ala	Leu Lys Gln Phe Arg	Leu Pro Leu Glu	
		1315	1320	
Glu Thr 1325	Glu Leu Glu Lys	Arg Ile Ile Val Asp	Asp Thr Ser Thr	
		1330	1335	
Gln Trp 1340	Ser Lys Asn Met	Lys His Leu Thr Pro	Ser Thr Leu Thr	
		1345	1350	
Gln Ile 1355	Asp Tyr Asn Glu	Lys Glu Lys Gly Ala	Ile Thr Gln Ser	
		1360	1365	
Pro Leu 1370	Ser Asp Cys Leu	Thr Arg Ser His Ser	Ile Pro Gln Ala	
		1375	1380	
Asn Arg 1385	Ser Pro Leu Pro	Ile Ala Lys Val Ser	Ser Phe Pro Ser	
		1390	1395	
Ile Arg 1400	Pro Ile Tyr Leu	Thr Arg Val Leu Phe	Gln Asp Asn Ser	
		1405	1410	

Ser	His	Leu	Pro	Ala	Ala	Ser	Tyr	Arg	Lys	Lys	Asp	Ser	Gly	Val
1415						1420					1425			
Gln	Glu	Ser	Ser	His	Phe	Leu	Gln	Gly	Ala	Lys	Lys	Asn	Asn	Leu
1430						1435					1440			
Ser	Leu	Ala	Ile	Leu	Thr	Leu	Glu	Met	Thr	Gly	Asp	Gln	Arg	Glu
1445						1450					1455			
Val	Gly	Ser	Leu	Gly	Thr	Ser	Ala	Thr	Asn	Ser	Val	Thr	Tyr	Lys
1460						1465					1470			
Lys	Val	Glu	Asn	Thr	Val	Leu	Pro	Lys	Pro	Asp	Leu	Pro	Lys	Thr
1475						1480					1485			
Ser	Gly	Lys	Val	Glu	Leu	Leu	Pro	Lys	Val	His	Ile	Tyr	Gln	Lys
1490						1495					1500			
Asp	Leu	Phe	Pro	Thr	Glu	Thr	Ser	Asn	Gly	Ser	Pro	Gly	His	Leu
1505						1510					1515			
Asp	Leu	Val	Glu	Gly	Ser	Leu	Leu	Gln	Gly	Thr	Glu	Gly	Ala	Ile
1520						1525					1530			
Lys	Trp	Asn	Glu	Ala	Asn	Arg	Pro	Gly	Lys	Val	Pro	Phe	Leu	Arg
1535						1540					1545			
Val	Ala	Thr	Glu	Ser	Ser	Ala	Lys	Thr	Pro	Ser	Lys	Leu	Leu	Asp
1550						1555					1560			
Pro	Leu	Ala	Trp	Asp	Asn	His	Tyr	Gly	Thr	Gln	Ile	Pro	Lys	Glu
1565						1570					1575			
Glu	Trp	Lys	Ser	Gln	Glu	Lys	Ser	Pro	Glu	Lys	Thr	Ala	Phe	Lys
1580						1585					1590			
Lys	Lys	Asp	Thr	Ile	Leu	Ser	Leu	Asn	Ala	Cys	Glu	Ser	Asn	His
1595						1600					1605			
Ala	Ile	Ala	Ala	Ile	Asn	Glu	Gly	Gln	Asn	Lys	Pro	Glu	Ile	Glu
1610						1615					1620			
Val	Thr	Trp	Ala	Lys	Gln	Gly	Arg	Thr	Glu	Arg	Leu	Cys	Ser	Gln
1625						1630					1635			
Asn	Pro	Pro	Val	Leu	Lys	Arg	His	Gln	Arg	Glu	Ile	Thr	Arg	Thr
1640						1645					1650			
Thr	Leu	Gln	Ser	Asp	Gln	Glu	Glu	Ile	Asp	Tyr	Asp	Asp	Thr	Ile
1655						1660					1665			
Ser	Val	Glu	Met	Lys	Lys	Glu	Asp	Phe	Asp	Ile	Tyr	Asp	Glu	Asp
1670						1675					1680			
Glu	Asn	Gln	Ser	Pro	Arg	Ser	Phe	Gln	Lys	Lys	Thr	Arg	His	Tyr
1685						1690					1695			

Phe Ile	Ala Ala Val	Glu Arg	Leu Trp Asp Tyr	Gly	Met Ser Ser
1700		1705		1710	
Ser Pro	His Val Leu Arg	Asn	Arg Ala Gln Ser	Gly	Ser Val Pro
1715		1720		1725	
Gln Phe	Lys Lys Val Val	Phe	Gln Glu Phe Thr	Asp	Gly Ser Phe
1730		1735		1740	
Thr Gln	Pro Leu Tyr Arg	Gly	Glu Leu Asn Glu His	Leu Gly Leu	
1745		1750		1755	
Leu Gly	Pro Tyr Ile Arg	Ala	Glu Val Glu Asp	Asn	Ile Met Val
1760		1765		1770	
Thr Phe	Arg Asn Gln Ala	Ser	Arg Pro Tyr Ser	Phe	Tyr Ser Ser
1775		1780		1785	
Leu Ile	Ser Tyr Glu Glu	Asp	Gln Arg Gln Gly	Ala	Glu Pro Arg
1790		1795		1800	
Lys Asn	Phe Val Lys Pro	Asn	Glu Thr Lys Thr	Tyr	Phe Trp Lys
1805		1810		1815	
Val Gln	His His Met Ala	Pro	Thr Lys Asp Glu	Phe	Asp Cys Lys
1820		1825		1830	
Ala Trp	Ala Tyr Phe Ser	Asp	Val Asp Leu Glu	Lys	Asp Val His
1835		1840		1845	
Ser Gly	Leu Ile Gly Pro	Leu	Leu Val Cys His	Thr	Asn Thr Leu
1850		1855		1860	
Asn Pro	Ala His Gly Arg	Gln	Val Thr Val Gln	Glu	Phe Ala Leu
1865		1870		1875	
Phe Phe	Thr Ile Phe Asp	Glu	Thr Lys Ser Trp	Tyr	Phe Thr Glu
1880		1885		1890	
Asn Met	Glu Arg Asn Cys	Arg	Ala Pro Cys Asn	Ile	Gln Met Glu
1895		1900		1905	
Asp Pro	Thr Phe Lys Glu	Asn	Tyr Arg Phe His	Ala	Ile Asn Gly
1910		1915		1920	
Tyr Ile	Met Asp Thr Leu	Pro	Gly Leu Val Met	Ala	Gln Asp Gln
1925		1930		1935	
Arg Ile	Arg Trp Tyr Leu	Leu	Ser Met Gly Ser	Asn	Glu Asn Ile
1940		1945		1950	
His Ser	Ile His Phe Ser	Gly	His Val Phe Thr	Val	Arg Lys Lys
1955		1960		1965	

Glu Glu 1970	Tyr Lys Met Ala Leu 1975	Tyr Asn Leu Tyr Pro 1980	Gly Val Phe
Glu Thr 1985	Val Glu Met Leu Pro 1990	Ser Lys Ala Gly Ile 1995	Trp Arg Val
Glu Cys 2000	Leu Ile Gly Glu His 2005	Leu His Ala Gly Met 2010	Ser Thr Leu
Phe Leu 2015	Val Tyr Ser Asn Lys 2020	Cys Gln Thr Pro Leu 2025	Gly Met Ala
Ser Gly 2030	His Ile Arg Asp Phe 2035	Gln Ile Thr Ala Ser 2040	Gly Gln Tyr
Gly Gln 2045	Trp Ala Pro Lys Leu 2050	Ala Arg Leu His Tyr 2055	Ser Gly Ser
Ile Asn 2060	Ala Trp Ser Thr Lys 2065	Glu Pro Phe Ser Trp 2070	Ile Lys Val
Asp Leu 2075	Leu Ala Pro Met Ile 2080	Ile His Gly Ile Lys 2085	Thr Gln Gly
Ala Arg 2090	Gln Lys Phe Ser Ser 2095	Leu Tyr Ile Ser Gln 2100	Phe Ile Ile
Met Tyr 2105	Ser Leu Asp Gly Lys 2110	Lys Trp Gln Thr Tyr 2115	Arg Gly Asn
Ser Thr 2120	Gly Thr Leu Met Val 2125	Phe Phe Gly Asn Val 2130	Asp Ser Ser
Gly Ile 2135	Lys His Asn Ile Phe 2140	Asn Pro Pro Ile Ile 2145	Ala Arg Tyr
Ile Arg 2150	Leu His Pro Thr His 2155	Tyr Ser Ile Arg Ser 2160	Thr Leu Arg
Met Glu 2165	Leu Met Gly Cys Asp 2170	Leu Asn Ser Cys Ser 2175	Met Pro Leu
Gly Met 2180	Glu Ser Lys Ala Ile 2185	Ser Asp Ala Gln Ile 2190	Thr Ala Ser
Ser Tyr 2195	Phe Thr Asn Met Phe 2200	Ala Thr Trp Ser Pro 2205	Ser Lys Ala
Arg Leu 2210	His Leu Gln Gly Arg 2215	Ser Asn Ala Trp Arg 2220	Pro Gln Val
Asn Asn 2225	Pro Lys Glu Trp Leu 2230	Gln Val Asp Phe Gln 2235	Lys Thr Met
Lys Val 2240	Thr Gly Val Thr Thr 2245	Gln Gly Val Lys Ser 2250	Leu Leu Thr

Ser Met Tyr Val Lys Glu Phe Leu Ile Ser Ser Ser Gln Asp Gly
2255 2260 2265

His Gln Trp Thr Leu Phe Phe Gln Asn Gly Lys Val Lys Val Phe
2270 2275 2280

Gln Gly Asn Gln Asp Ser Phe Thr Pro Val Val Asn Ser Leu Asp
2285 2290 2295

Pro Pro Leu Leu Thr Arg Tyr Leu Arg Ile His Pro Gln Ser Trp
2300 2305 2310

Val His Gln Ile Ala Leu Arg Met Glu Val Leu Gly Cys Glu Ala
2315 2320 2325

Gln Asp Leu Tyr
2330

<210> 15

<211> 1444

<212> PRT

<213> Artificial sequence

<220>

<223> mature single chain Factor VIII

<400> 15

Ala Thr Arg Arg Tyr Tyr Leu Gly Ala Val Glu Leu Ser Trp Asp Tyr
1 5 10 15

Met Gln Ser Asp Leu Gly Glu Leu Pro Val Asp Ala Arg Phe Pro Pro
20 25 30

Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val Tyr Lys Lys
35 40 45

Thr Leu Phe Val Glu Phe Thr Asp His Leu Phe Asn Ile Ala Lys Pro
50 55 60

Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln Ala Glu Val
65 70 75 80

Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser His Pro Val
85 90 95

Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser Glu Gly Ala
100 105 110

Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys Glu Asp Asp Lys Val
115 120 125

Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu Lys Glu Asn
130 135 140

Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser Tyr Leu Ser
145 150 155 160

His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile Gly Ala Leu
 165 170 175

Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr Gln Thr Leu
 180 185 190

His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly Lys Ser Trp
 195 200 205

His Ser Glu Thr Lys Asn Ser Leu Met Gln Asp Arg Asp Ala Ala Ser
 210 215 220

Ala Arg Ala Trp Pro Lys Met His Thr Val Asn Gly Tyr Val Asn Arg
 225 230 235 240

Ser Leu Pro Gly Leu Ile Gly Cys His Arg Lys Ser Val Tyr Trp His
 245 250 255

Val Ile Gly Met Gly Thr Thr Pro Glu Val His Ser Ile Phe Leu Glu
 260 265 270

Gly His Thr Phe Leu Val Arg Asn His Arg Gln Ala Ser Leu Glu Ile
 275 280 285

Ser Pro Ile Thr Phe Leu Thr Ala Gln Thr Leu Leu Met Asp Leu Gly
 290 295 300

Gln Phe Leu Leu Phe Cys His Ile Ser Ser His Gln His Asp Gly Met
 305 310 315 320

Glu Ala Tyr Val Lys Val Asp Ser Cys Pro Glu Glu Pro Gln Leu Arg
 325 330 335

Met Lys Asn Asn Glu Glu Ala Glu Asp Tyr Asp Asp Asp Leu Thr Asp
 340 345 350

Ser Glu Met Asp Val Val Arg Phe Asp Asp Asp Asn Ser Pro Ser Phe
 355 360 365

Ile Gln Ile Arg Ser Val Ala Lys Lys His Pro Lys Thr Trp Val His
 370 375 380

Tyr Ile Ala Ala Glu Glu Glu Asp Trp Asp Tyr Ala Pro Leu Val Leu
 385 390 395 400

Ala Pro Asp Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn Asn Gly Pro
 405 410 415

Gln Arg Ile Gly Arg Lys Tyr Lys Lys Val Arg Phe Met Ala Tyr Thr
 420 425 430

Asp Glu Thr Phe Lys Thr Arg Glu Ala Ile Gln His Glu Ser Gly Ile
 435 440 445

Leu Gly Pro Leu Leu Tyr Gly Glu Val Gly Asp Thr Leu Leu Ile Ile
 450 455 460

Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn Ile Tyr Pro His Gly Ile
 465 470 475 480
 Thr Asp Val Arg Pro Leu Tyr Ser Arg Arg Leu Pro Lys Gly Val Lys
 485 490 495
 His Leu Lys Asp Phe Pro Ile Leu Pro Gly Glu Ile Phe Lys Tyr Lys
 500 505 510
 Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys Ser Asp Pro Arg Cys
 515 520 525
 Leu Thr Arg Tyr Tyr Ser Ser Phe Val Asn Met Glu Arg Asp Leu Ala
 530 535 540
 Ser Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu Ser Val Asp
 545 550 555 560
 Gln Arg Gly Asn Gln Ile Met Ser Asp Lys Arg Asn Val Ile Leu Phe
 565 570 575
 Ser Val Phe Asp Glu Asn Arg Ser Trp Tyr Leu Thr Glu Asn Ile Gln
 580 585 590
 Arg Phe Leu Pro Asn Pro Ala Gly Val Gln Leu Glu Asp Pro Glu Phe
 595 600 605
 Gln Ala Ser Asn Ile Met His Ser Ile Asn Gly Tyr Val Phe Asp Ser
 610 615 620
 Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp Tyr Ile Leu
 625 630 635 640
 Ser Ile Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe Ser Gly Tyr
 645 650 655
 Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr Leu Phe Pro
 660 665 670
 Phe Ser Gly Glu Thr Val Phe Met Ser Met Glu Asn Pro Gly Leu Trp
 675 680 685
 Ile Leu Gly Cys His Asn Ser Asp Phe Arg Asn Arg Gly Met Thr Ala
 690 695 700
 Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr Gly Asp Tyr Tyr Glu
 705 710 715 720
 Asp Ser Tyr Glu Asp Ile Ser Ala Tyr Leu Leu Ser Lys Asn Asn Ala
 725 730 735
 Ile Glu Pro Arg Ser Phe Ser Gln Asn Ser Arg His Arg Ser Thr Arg
 740 745 750
 Gln Lys Gln Phe Asn Ala Thr Thr Ile Pro Glu Asn Thr Thr Leu Gln
 755 760 765

Ser Asp Gln Glu Glu Ile Asp Tyr Asp Asp Thr Ile Ser Val Glu Met
 770 775 780

Lys Lys Glu Asp Phe Asp Ile Tyr Asp Glu Asp Glu Asn Gln Ser Pro
 785 790 795 800

Arg Ser Phe Gln Lys Lys Thr Arg His Tyr Phe Ile Ala Ala Val Glu
 805 810 815

Arg Leu Trp Asp Tyr Gly Met Ser Ser Ser Pro His Val Leu Arg Asn
 820 825 830

Arg Ala Gln Ser Gly Ser Val Pro Gln Phe Lys Lys Val Val Phe Gln
 835 840 845

Glu Phe Thr Asp Gly Ser Phe Thr Gln Pro Leu Tyr Arg Gly Glu Leu
 850 855 860

Asn Glu His Leu Gly Leu Leu Gly Pro Tyr Ile Arg Ala Glu Val Glu
 865 870 875 880

Asp Asn Ile Met Val Thr Phe Arg Asn Gln Ala Ser Arg Pro Tyr Ser
 885 890 895

Phe Tyr Ser Ser Leu Ile Ser Tyr Glu Glu Asp Gln Arg Gln Gly Ala
 900 905 910

Glu Pro Arg Lys Asn Phe Val Lys Pro Asn Glu Thr Lys Thr Tyr Phe
 915 920 925

Trp Lys Val Gln His His Met Ala Pro Thr Lys Asp Glu Phe Asp Cys
 930 935 940

Lys Ala Trp Ala Tyr Phe Ser Asp Val Asp Leu Glu Lys Asp Val His
 945 950 955 960

Ser Gly Leu Ile Gly Pro Leu Leu Val Cys His Thr Asn Thr Leu Asn
 965 970 975

Pro Ala His Gly Arg Gln Val Thr Val Gln Glu Phe Ala Leu Phe Phe
 980 985 990

Thr Ile Phe Asp Glu Thr Lys Ser Trp Tyr Phe Thr Glu Asn Met Glu
 995 1000 1005

Arg Asn Cys Arg Ala Pro Cys Asn Ile Gln Met Glu Asp Pro Thr
 1010 1015 1020

Phe Lys Glu Asn Tyr Arg Phe His Ala Ile Asn Gly Tyr Ile Met
 1025 1030 1035

Asp Thr Leu Pro Gly Leu Val Met Ala Gln Asp Gln Arg Ile Arg
 1040 1045 1050

Trp	Tyr	Leu	Leu	Ser	Met	Gly	Ser	Asn	Glu	Asn	Ile	His	Ser	Ile
1055						1060					1065			
His	Phe	Ser	Gly	His	Val	Phe	Thr	Val	Arg	Lys	Lys	Glu	Glu	Tyr
1070						1075					1080			
Lys	Met	Ala	Leu	Tyr	Asn	Leu	Tyr	Pro	Gly	Val	Phe	Glu	Thr	Val
1085						1090					1095			
Glu	Met	Leu	Pro	Ser	Lys	Ala	Gly	Ile	Trp	Arg	Val	Glu	Cys	Leu
1100						1105					1110			
Ile	Gly	Glu	His	Leu	His	Ala	Gly	Met	Ser	Thr	Leu	Phe	Leu	Val
1115						1120					1125			
Tyr	Ser	Asn	Lys	Cys	Gln	Thr	Pro	Leu	Gly	Met	Ala	Ser	Gly	His
1130						1135					1140			
Ile	Arg	Asp	Phe	Gln	Ile	Thr	Ala	Ser	Gly	Gln	Tyr	Gly	Gln	Trp
1145						1150					1155			
Ala	Pro	Lys	Leu	Ala	Arg	Leu	His	Tyr	Ser	Gly	Ser	Ile	Asn	Ala
1160						1165					1170			
Trp	Ser	Thr	Lys	Glu	Pro	Phe	Ser	Trp	Ile	Lys	Val	Asp	Leu	Leu
1175						1180					1185			
Ala	Pro	Met	Ile	Ile	His	Gly	Ile	Lys	Thr	Gln	Gly	Ala	Arg	Gln
1190						1195					1200			
Lys	Phe	Ser	Ser	Leu	Tyr	Ile	Ser	Gln	Phe	Ile	Ile	Met	Tyr	Ser
1205						1210					1215			
Leu	Asp	Gly	Lys	Lys	Trp	Gln	Thr	Tyr	Arg	Gly	Asn	Ser	Thr	Gly
1220						1225					1230			
Thr	Leu	Met	Val	Phe	Phe	Gly	Asn	Val	Asp	Ser	Ser	Gly	Ile	Lys
1235						1240					1245			
His	Asn	Ile	Phe	Asn	Pro	Pro	Ile	Ile	Ala	Arg	Tyr	Ile	Arg	Leu
1250						1255					1260			
His	Pro	Thr	His	Tyr	Ser	Ile	Arg	Ser	Thr	Leu	Arg	Met	Glu	Leu
1265						1270					1275			
Met	Gly	Cys	Asp	Leu	Asn	Ser	Cys	Ser	Met	Pro	Leu	Gly	Met	Glu
1280						1285					1290			
Ser	Lys	Ala	Ile	Ser	Asp	Ala	Gln	Ile	Thr	Ala	Ser	Ser	Tyr	Phe
1295						1300					1305			
Thr	Asn	Met	Phe	Ala	Thr	Trp	Ser	Pro	Ser	Lys	Ala	Arg	Leu	His
1310						1315					1320			
Leu	Gln	Gly	Arg	Ser	Asn	Ala	Trp	Arg	Pro	Gln	Val	Asn	Asn	Pro
1325						1330					1335			

Lys Glu Trp Leu Gln Val Asp Phe Gln Lys Thr Met Lys Val Thr
1340 1345 1350

Gly Val Thr Thr Gln Gly Val Lys Ser Leu Leu Thr Ser Met Tyr
1355 1360 1365

Val Lys Glu Phe Leu Ile Ser Ser Ser Gln Asp Gly His Gln Trp
1370 1375 1380

Thr Leu Phe Phe Gln Asn Gly Lys Val Lys Val Phe Gln Gly Asn
1385 1390 1395

Gln Asp Ser Phe Thr Pro Val Val Asn Ser Leu Asp Pro Pro Leu
1400 1405 1410

Leu Thr Arg Tyr Leu Arg Ile His Pro Gln Ser Trp Val His Gln
1415 1420 1425

Ile Ala Leu Arg Met Glu Val Leu Gly Cys Glu Ala Gln Asp Leu
1430 1435 1440

Tyr

<210> 16

<211> 585

<212> PRT

<213> Homo sapiens

<400> 16

Asp Ala His Lys Ser Glu Val Ala His Arg Phe Lys Asp Leu Gly Glu
1 5 10 15

Glu Asn Phe Lys Ala Leu Val Leu Ile Ala Phe Ala Gln Tyr Leu Gln
20 25 30

Gln Cys Pro Phe Glu Asp His Val Lys Leu Val Asn Glu Val Thr Glu
35 40 45

Phe Ala Lys Thr Cys Val Ala Asp Glu Ser Ala Glu Asn Cys Asp Lys
50 55 60

Ser Leu His Thr Leu Phe Gly Asp Lys Leu Cys Thr Val Ala Thr Leu
65 70 75 80

Arg Glu Thr Tyr Gly Glu Met Ala Asp Cys Cys Ala Lys Gln Glu Pro
85 90 95

Glu Arg Asn Glu Cys Phe Leu Gln His Lys Asp Asp Asn Pro Asn Leu
100 105 110

Pro Arg Leu Val Arg Pro Glu Val Asp Val Met Cys Thr Ala Phe His
115 120 125

Asp Asn Glu Glu Thr Phe Leu Lys Lys Tyr Leu Tyr Glu Ile Ala Arg
130 135 140

Arg His Pro Tyr Phe Tyr Ala Pro Glu Leu Leu Phe Phe Ala Lys Arg
 145 150 155 160
 Tyr Lys Ala Ala Phe Thr Glu Cys Cys Gln Ala Ala Asp Lys Ala Ala
 165 170 175
 Cys Leu Leu Pro Lys Leu Asp Glu Leu Arg Asp Glu Gly Lys Ala Ser
 180 185 190
 Ser Ala Lys Gln Arg Leu Lys Cys Ala Ser Leu Gln Lys Phe Gly Glu
 195 200 205
 Arg Ala Phe Lys Ala Trp Ala Val Ala Arg Leu Ser Gln Arg Phe Pro
 210 215 220
 Lys Ala Glu Phe Ala Glu Val Ser Lys Leu Val Thr Asp Leu Thr Lys
 225 230 235 240
 Val His Thr Glu Cys Cys His Gly Asp Leu Leu Glu Cys Ala Asp Asp
 245 250 255
 Arg Ala Asp Leu Ala Lys Tyr Ile Cys Glu Asn Gln Asp Ser Ile Ser
 260 265 270
 Ser Lys Leu Lys Glu Cys Cys Glu Lys Pro Leu Leu Glu Lys Ser His
 275 280 285
 Cys Ile Ala Glu Val Glu Asn Asp Glu Met Pro Ala Asp Leu Pro Ser
 290 295 300
 Leu Ala Ala Asp Phe Val Glu Ser Lys Asp Val Cys Lys Asn Tyr Ala
 305 310 315 320
 Glu Ala Lys Asp Val Phe Leu Gly Met Phe Leu Tyr Glu Tyr Ala Arg
 325 330 335
 Arg His Pro Asp Tyr Ser Val Val Leu Leu Leu Arg Leu Ala Lys Thr
 340 345 350
 Tyr Glu Thr Thr Leu Glu Lys Cys Cys Ala Ala Ala Asp Pro His Glu
 355 360 365
 Cys Tyr Ala Lys Val Phe Asp Glu Phe Lys Pro Leu Val Glu Glu Pro
 370 375 380
 Gln Asn Leu Ile Lys Gln Asn Cys Glu Leu Phe Glu Gln Leu Gly Glu
 385 390 395 400
 Tyr Lys Phe Gln Asn Ala Leu Leu Val Arg Tyr Thr Lys Lys Val Pro
 405 410 415
 Gln Val Ser Thr Pro Thr Leu Val Glu Val Ser Arg Asn Leu Gly Lys
 420 425 430
 Val Gly Ser Lys Cys Cys Lys His Pro Glu Ala Lys Arg Met Pro Cys
 435 440 445

Ala Glu Asp Tyr Leu Ser Val Val Leu Asn Gln Leu Cys Val Leu His
450 455 460

Glu Lys Thr Pro Val Ser Asp Arg Val Thr Lys Cys Cys Thr Glu Ser
465 470 475 480

Leu Val Asn Arg Arg Pro Cys Phe Ser Ala Leu Glu Val Asp Glu Thr
485 490 495

Tyr Val Pro Lys Glu Phe Asn Ala Glu Thr Phe Thr Phe His Ala Asp
500 505 510

Ile Cys Thr Leu Ser Glu Lys Glu Arg Gln Ile Lys Lys Gln Thr Ala
515 520 525

Leu Val Glu Leu Val Lys His Lys Pro Lys Ala Thr Lys Glu Gln Leu
530 535 540

Lys Ala Val Met Asp Asp Phe Ala Ala Phe Val Glu Lys Cys Cys Lys
545 550 555 560

Ala Asp Asp Lys Glu Thr Cys Phe Ala Glu Glu Gly Lys Lys Leu Val
565 570 575

Ala Ala Ser Gln Ala Ala Leu Gly Leu

580 585

<210> 17

<211> 479

<212> PRT

<213> Artificial sequence

<220>

<223> Modified D'-D3 domain of VWFPR

<400> 17

Ser Leu Tyr Cys Arg Pro Pro Met Val Lys Leu Val Cys Pro Ala Asp
1 5 10 15

Asn Leu Arg Ala Glu Gly Leu Glu Cys Thr Lys Thr Cys Gln Asn Tyr
20 25 30

Asp Leu Glu Cys Met Ser Met Gly Cys Val Ser Gly Cys Leu Cys Pro
35 40 45

Pro Gly Met Val Arg His Glu Asn Arg Cys Val Ala Leu Glu Arg Cys
50 55 60

Pro Cys Phe His Gln Gly Lys Glu Tyr Ala Pro Gly Glu Thr Val Lys
65 70 75 80

Ile Gly Cys Asn Thr Cys Val Cys Arg Asp Arg Lys Trp Asn Cys Thr
85 90 95

Asp His Val Cys Asp Ala Thr Cys Ser Thr Ile Gly Met Ala His Tyr
 100 105 110

Leu Thr Phe Asp Gly Leu Lys Tyr Leu Phe Pro Gly Glu Cys Gln Tyr
 115 120 125

Val Leu Val Gln Asp Tyr Cys Gly Ser Asn Pro Gly Thr Phe Arg Ile
 130 135 140

Leu Val Gly Asn Lys Gly Cys Ser His Pro Ser Val Lys Cys Lys Lys
 145 150 155 160

Arg Val Thr Ile Leu Val Glu Gly Gly Glu Ile Glu Leu Phe Asp Gly
 165 170 175

Glu Val Asn Val Lys Arg Pro Met Lys Asp Glu Thr His Phe Glu Val
 180 185 190

Val Glu Ser Gly Arg Tyr Ile Ile Leu Leu Leu Gly Lys Ala Leu Ser
 195 200 205

Val Val Trp Asp Arg His Leu Ser Ile Ser Val Val Leu Lys Gln Thr
 210 215 220

Tyr Gln Glu Lys Val Cys Gly Leu Cys Gly Asn Phe Asp Gly Ile Gln
 225 230 235 240

Asn Asn Asp Leu Thr Ser Ser Asn Leu Gln Val Glu Glu Asp Pro Val
 245 250 255

Asp Phe Gly Asn Ser Trp Lys Val Ser Ser Gln Cys Ala Asp Thr Arg
 260 265 270

Lys Val Pro Leu Asp Ser Ser Pro Ala Thr Cys His Asn Asn Ile Met
 275 280 285

Lys Gln Thr Met Val Asp Ser Ser Cys Arg Ile Leu Thr Ser Asp Val
 290 295 300

Phe Gln Asp Cys Asn Lys Leu Val Asp Pro Glu Pro Tyr Leu Asp Ala
 305 310 315 320

Cys Ile Tyr Asp Thr Cys Ser Cys Glu Ser Ile Gly Asp Cys Ala Cys
 325 330 335

Phe Cys Asp Thr Ile Ala Ala Tyr Ala His Val Cys Ala Gln His Gly
 340 345 350

Lys Val Val Thr Trp Arg Thr Ala Thr Leu Cys Pro Gln Ser Cys Glu
 355 360 365

Glu Arg Asn Leu Arg Glu Asn Gly Tyr Glu Cys Glu Trp Arg Tyr Asn
 370 375 380

Ser Cys Ala Pro Ala Cys Gln Val Thr Cys Gln His Pro Glu Pro Leu

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385          390          395          400
Ala Cys Pro Val Gln Cys Val Glu Gly Cys His Ala His Cys Pro Pro
      405                                410                                415
Gly Lys Ile Leu Asp Glu Leu Leu Gln Thr Cys Val Asp Pro Glu Asp
      420                                425                                430
Cys Pro Val Cys Glu Val Ala Gly Arg Arg Phe Ala Ser Gly Lys Lys
      435                                440                                445
Val Thr Leu Asn Pro Ser Asp Pro Glu His Cys Gln Ile Cys His Cys
      450                                455                                460
Asp Val Val Asn Leu Thr Cys Glu Ala Cys Gln Glu Pro Gly Gly
      465                                470                                475
<210> 18
<211> 479
<212> PRT
<213> Artificial sequence
<220>
<223> Modified D'-D3 domain of VWFPRT
<400> 18
Gly Leu Tyr Cys Arg Pro Pro Met Val Lys Leu Val Cys Pro Ala Asp
1      5      10      15
Asn Leu Arg Ala Glu Gly Leu Glu Cys Thr Lys Thr Cys Gln Asn Tyr
      20      25      30
Asp Leu Glu Cys Met Ser Met Gly Cys Val Ser Gly Cys Leu Cys Pro
      35      40      45
Pro Gly Met Val Arg His Glu Asn Arg Cys Val Ala Leu Glu Arg Cys
      50      55      60
Pro Cys Phe His Gln Gly Lys Glu Tyr Ala Pro Gly Glu Thr Val Lys
      65      70      75      80
Ile Gly Cys Asn Thr Cys Val Cys Arg Asp Arg Lys Trp Asn Cys Thr
      85      90      95
Asp His Val Cys Asp Ala Thr Cys Ser Thr Ile Gly Met Ala His Tyr
      100      105      110
Leu Thr Phe Asp Gly Leu Lys Tyr Leu Phe Pro Gly Glu Cys Gln Tyr
      115      120      125
Val Leu Val Gln Asp Tyr Cys Gly Ser Asn Pro Gly Thr Phe Arg Ile
      130      135      140

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Leu Val Gly Asn Lys Gly Cys Ser His Pro Ser Val Lys Cys Lys Lys
 145 150 155 160

Arg Val Thr Ile Leu Val Glu Gly Gly Glu Ile Glu Leu Phe Asp Gly
 165 170 175

Glu Val Asn Val Lys Arg Pro Met Lys Asp Glu Thr His Phe Glu Val
 180 185 190

Val Glu Ser Gly Arg Tyr Ile Ile Leu Leu Leu Gly Lys Ala Leu Ser
 195 200 205

Val Val Trp Asp Arg His Leu Ser Ile Ser Val Val Leu Lys Gln Thr
 210 215 220

Tyr Gln Glu Lys Val Cys Gly Leu Cys Gly Asn Phe Asp Gly Ile Gln
 225 230 235 240

Asn Asn Asp Leu Thr Ser Ser Asn Leu Gln Val Glu Glu Asp Pro Val
 245 250 255

Asp Phe Gly Asn Ser Trp Lys Val Ser Ser Gln Cys Ala Asp Thr Arg
 260 265 270

Lys Val Pro Leu Asp Ser Ser Pro Ala Thr Cys His Asn Asn Ile Met
 275 280 285

Lys Gln Thr Met Val Asp Ser Ser Cys Arg Ile Leu Thr Ser Asp Val
 290 295 300

Phe Gln Asp Cys Asn Lys Leu Val Asp Pro Glu Pro Tyr Leu Asp Val
 305 310 315 320

Cys Ile Tyr Asp Thr Cys Ser Cys Glu Ser Ile Gly Asp Cys Ala Cys
 325 330 335

Phe Cys Asp Thr Ile Ala Ala Tyr Ala His Val Cys Ala Gln His Gly
 340 345 350

Lys Val Val Thr Trp Arg Thr Ala Thr Leu Cys Pro Gln Ser Cys Glu
 355 360 365

Glu Arg Asn Leu Arg Glu Asn Gly Tyr Glu Cys Glu Trp Arg Tyr Asn
 370 375 380

Ser Cys Ala Pro Ala Cys Gln Val Thr Cys Gln His Pro Glu Pro Leu
 385 390 395 400

Ala Cys Pro Val Gln Cys Val Glu Gly Cys His Ala His Cys Pro Pro
 405 410 415

Gly Lys Ile Leu Asp Glu Leu Leu Gln Thr Cys Val Asp Pro Glu Asp
 420 425 430

Cys Pro Val Cys Glu Val Ala Gly Arg Arg Phe Ala Ser Gly Lys Lys

Val Thr Leu Asn Pro Ser Asp Pro Glu His Cys Gln Ile Cys His Cys
450 455 460

Asp Val Val Asn Leu Thr Cys Glu Ala Cys Gln Glu Pro Gly Gly
465 470 475

<210> 19

<211> 479

<212> PRT

<213> Artificial sequence

<220>

<223> Modified D'-D3 domain of VWFPR

<400> 19

Pro Leu Ile Cys Arg Pro Pro Met Val Lys Leu Val Cys Pro Ala Asp
1 5 10 15

Asn Leu Arg Ala Glu Gly Leu Glu Cys Thr Lys Thr Cys Gln Asn Tyr
20 25 30

Asp Leu Glu Cys Met Ser Met Gly Cys Val Ser Gly Cys Leu Cys Pro
35 40 45

Pro Gly Met Val Arg His Glu Asn Arg Cys Val Ala Leu Glu Arg Cys
50 55 60

Pro Cys Phe His Gln Gly Lys Glu Tyr Ala Pro Gly Glu Thr Val Lys
65 70 75 80

Ile Gly Cys Asn Thr Cys Val Cys Arg Asp Arg Lys Trp Asn Cys Thr
85 90 95

Asp His Val Cys Asp Ala Thr Cys Ser Thr Ile Gly Met Ala His Tyr
100 105 110

Leu Thr Phe Asp Gly Leu Lys Tyr Leu Phe Pro Gly Glu Cys Gln Tyr
115 120 125

Val Leu Val Gln Asp Tyr Cys Gly Ser Asn Pro Gly Thr Phe Arg Ile
130 135 140

Leu Val Gly Asn Lys Gly Cys Ser His Pro Ser Val Lys Cys Lys Lys
145 150 155 160

Arg Val Thr Ile Leu Val Glu Gly Gly Glu Ile Glu Leu Phe Asp Gly
165 170 175

Glu Val Asn Val Lys Arg Pro Met Lys Asp Glu Thr His Phe Glu Val
180 185 190

Val Glu Ser Gly Arg Tyr Ile Ile Leu Leu Leu Gly Lys Ala Leu Ser
195 200 205

Val Val Trp Asp Arg His Leu Ser Ile Ser Val Val Leu Lys Gln Thr
 210 215 220
 Tyr Gln Glu Lys Val Cys Gly Leu Cys Gly Asn Phe Asp Gly Ile Gln
 225 230 235 240
 Asn Asn Asp Leu Thr Ser Ser Asn Leu Gln Val Glu Glu Asp Pro Val
 245 250 255
 Asp Phe Gly Asn Ser Trp Lys Val Ser Ser Gln Cys Ala Asp Thr Arg
 260 265 270
 Lys Val Pro Leu Asp Ser Ser Pro Ala Thr Cys His Asn Asn Ile Met
 275 280 285
 Lys Gln Thr Met Val Asp Ser Ser Cys Arg Ile Leu Thr Ser Asp Val
 290 295 300
 Phe Gln Asp Cys Asn Lys Leu Val Asp Pro Glu Pro Tyr Leu Asp Val
 305 310 315 320
 Cys Ile Tyr Asp Thr Cys Ser Cys Glu Ser Ile Gly Asp Cys Ala Cys
 325 330 335
 Phe Cys Asp Thr Ile Ala Ala Tyr Ala His Val Cys Ala Gln His Gly
 340 345 350
 Lys Val Val Thr Trp Arg Thr Ala Thr Leu Cys Pro Gln Ser Cys Glu
 355 360 365
 Glu Arg Asn Leu Arg Glu Asn Gly Tyr Glu Cys Glu Trp Arg Tyr Asn
 370 375 380
 Ser Cys Ala Pro Ala Cys Gln Val Thr Cys Gln His Pro Glu Pro Leu
 385 390 395 400
 Ala Cys Pro Val Gln Cys Val Glu Gly Cys His Ala His Cys Pro Pro
 405 410 415
 Gly Lys Ile Leu Asp Glu Leu Leu Gln Thr Cys Val Asp Pro Glu Asp
 420 425 430
 Cys Pro Val Cys Glu Val Ala Gly Arg Arg Phe Ala Ser Gly Lys Lys
 435 440 445
 Val Thr Leu Asn Pro Ser Asp Pro Glu His Cys Gln Ile Cys His Cys
 450 455 460
 Asp Val Val Asn Leu Thr Cys Glu Ala Cys Gln Glu Pro Gly Gly
 465 470 475

<210> 20

<211> 479

<212> PRT

<213> Artificial sequence

<220>

<223> Modified D'-D3 domain of VWFPR

<400> 20

Pro Leu Met Cys Arg Pro Pro Met Val Lys Leu Val Cys Pro Ala Asp
 1 5 10 15

Asn Leu Arg Ala Glu Gly Leu Glu Cys Thr Lys Thr Cys Gln Asn Tyr
 20 25 30

Asp Leu Glu Cys Met Ser Met Gly Cys Val Ser Gly Cys Leu Cys Pro
 35 40 45

Pro Gly Met Val Arg His Glu Asn Arg Cys Val Ala Leu Glu Arg Cys
 50 55 60

Pro Cys Phe His Gln Gly Lys Glu Tyr Ala Pro Gly Glu Thr Val Lys
 65 70 75 80

Ile Gly Cys Asn Thr Cys Val Cys Arg Asp Arg Lys Trp Asn Cys Thr
 85 90 95

Asp His Val Cys Asp Ala Thr Cys Ser Thr Ile Gly Met Ala His Tyr
 100 105 110

Leu Thr Phe Asp Gly Leu Lys Tyr Leu Phe Pro Gly Glu Cys Gln Tyr
 115 120 125

Val Leu Val Gln Asp Tyr Cys Gly Ser Asn Pro Gly Thr Phe Arg Ile
 130 135 140

Leu Val Gly Asn Lys Gly Cys Ser His Pro Ser Val Lys Cys Lys Lys
 145 150 155 160

Arg Val Thr Ile Leu Val Glu Gly Gly Glu Ile Glu Leu Phe Asp Gly
 165 170 175

Glu Val Asn Val Lys Arg Pro Met Lys Asp Glu Thr His Phe Glu Val
 180 185 190

Val Glu Ser Gly Arg Tyr Ile Ile Leu Leu Leu Gly Lys Ala Leu Ser
 195 200 205

Val Val Trp Asp Arg His Leu Ser Ile Ser Val Val Leu Lys Gln Thr
 210 215 220

Tyr Gln Glu Lys Val Cys Gly Leu Cys Gly Asn Phe Asp Gly Ile Gln
 225 230 235 240

Asn Asn Asp Leu Thr Ser Ser Asn Leu Gln Val Glu Glu Asp Pro Val
 245 250 255

Asp Phe Gly Asn Ser Trp Lys Val Ser Ser Gln Cys Ala Asp Thr Arg
 260 265 270

Lys Val Pro Leu Asp Ser Ser Pro Ala Thr Cys His Asn Asn Ile Met
 275 280 285

Lys Gln Thr Met Val Asp Ser Ser Cys Arg Ile Leu Thr Ser Asp Val
 290 295 300

Phe Gln Asp Cys Asn Lys Leu Val Asp Pro Glu Pro Tyr Leu Asp Val
 305 310 315 320

Cys Ile Tyr Asp Thr Cys Ser Cys Glu Ser Ile Gly Asp Cys Ala Cys
 325 330 335

Phe Cys Asp Thr Ile Ala Ala Tyr Ala His Val Cys Ala Gln His Gly
 340 345 350

Lys Val Val Thr Trp Arg Thr Ala Thr Leu Cys Pro Gln Ser Cys Glu
 355 360 365

Glu Arg Asn Leu Arg Glu Asn Gly Tyr Glu Cys Glu Trp Arg Tyr Asn
 370 375 380

Ser Cys Ala Pro Ala Cys Gln Val Thr Cys Gln His Pro Glu Pro Leu
 385 390 395 400

Ala Cys Pro Val Gln Cys Val Glu Gly Cys His Ala His Cys Pro Pro
 405 410 415

Gly Lys Ile Leu Asp Glu Leu Leu Gln Thr Cys Val Asp Pro Glu Asp
 420 425 430

Cys Pro Val Cys Glu Val Ala Gly Arg Arg Phe Ala Ser Gly Lys Lys
 435 440 445

Val Thr Leu Asn Pro Ser Asp Pro Glu His Cys Gln Ile Cys His Cys
 450 455 460

Asp Val Val Asn Leu Thr Cys Glu Ala Cys Gln Glu Pro Gly Gly
 465 470 475

<210> 21

<211> 479

<212> PRT

<213> Artificial sequence

<220>

<223> Modified D'-D3 domain of VWFPR

<400> 21

Val Leu Tyr Cys Arg Pro Pro Met Val Lys Leu Val Cys Pro Ala Asp
 1 5 10 15

Asn Leu Arg Ala Glu Gly Leu Glu Cys Thr Lys Thr Cys Gln Asn Tyr
20 25 30

Asp Leu Glu Cys Met Ser Met Gly Cys Val Ser Gly Cys Leu Cys Pro
35 40 45

Pro Gly Met Val Arg His Glu Asn Arg Cys Val Ala Leu Glu Arg Cys
50 55 60

Pro Cys Phe His Gln Gly Lys Glu Tyr Ala Pro Gly Glu Thr Val Lys
65 70 75 80

Ile Gly Cys Asn Thr Cys Val Cys Arg Asp Arg Lys Trp Asn Cys Thr
85 90 95

Asp His Val Cys Asp Ala Thr Cys Ser Thr Ile Gly Met Ala His Tyr
100 105 110

Leu Thr Phe Asp Gly Leu Lys Tyr Leu Phe Pro Gly Glu Cys Gln Tyr
115 120 125

Val Leu Val Gln Asp Tyr Cys Gly Ser Asn Pro Gly Thr Phe Arg Ile
130 135 140

Leu Val Gly Asn Lys Gly Cys Ser His Pro Ser Val Lys Cys Lys Lys
145 150 155 160

Arg Val Thr Ile Leu Val Glu Gly Gly Glu Ile Glu Leu Phe Asp Gly
165 170 175

Glu Val Asn Val Lys Arg Pro Met Lys Asp Glu Thr His Phe Glu Val
180 185 190

Val Glu Ser Gly Arg Tyr Ile Ile Leu Leu Leu Gly Lys Ala Leu Ser
195 200 205

Val Val Trp Asp Arg His Leu Ser Ile Ser Val Val Leu Lys Gln Thr
210 215 220

Tyr Gln Glu Lys Val Cys Gly Leu Cys Gly Asn Phe Asp Gly Ile Gln
225 230 235 240

Asn Asn Asp Leu Thr Ser Ser Asn Leu Gln Val Glu Glu Asp Pro Val
245 250 255

Asp Phe Gly Asn Ser Trp Lys Val Ser Ser Gln Cys Ala Asp Thr Arg
260 265 270

Lys Val Pro Leu Asp Ser Ser Pro Ala Thr Cys His Asn Asn Ile Met
275 280 285

Lys Gln Thr Met Val Asp Ser Ser Cys Arg Ile Leu Thr Ser Asp Val
290 295 300

Phe Gln Asp Cys Asn Lys Leu Val Asp Pro Glu Pro Tyr Leu Asp Val
305 310 315 320

Cys Ile Tyr Asp Thr Cys Ser Cys Glu Ser Ile Gly Asp Cys Ala Cys
 325 330 335

Phe Cys Asp Thr Ile Ala Ala Tyr Ala His Val Cys Ala Gln His Gly
 340 345 350

Lys Val Val Thr Trp Arg Thr Ala Thr Leu Cys Pro Gln Ser Cys Glu
 355 360 365

Glu Arg Asn Leu Arg Glu Asn Gly Tyr Glu Cys Glu Trp Arg Tyr Asn
 370 375 380

Ser Cys Ala Pro Ala Cys Gln Val Thr Cys Gln His Pro Glu Pro Leu

385 390 395 400

Ala Cys Pro Val Gln Cys Val Glu Gly Cys His Ala His Cys Pro Pro
 405 410 415

Gly Lys Ile Leu Asp Glu Leu Leu Gln Thr Cys Val Asp Pro Glu Asp
 420 425 430

Cys Pro Val Cys Glu Val Ala Gly Arg Arg Phe Ala Ser Gly Lys Lys
 435 440 445

Val Thr Leu Asn Pro Ser Asp Pro Glu His Cys Gln Ile Cys His Cys
 450 455 460

Asp Val Val Asn Leu Thr Cys Glu Ala Cys Gln Glu Pro Gly Gly
 465 470 475

<210> 22

<211> 479

<212> PRT

<213> Artificial sequence

<220>

<223> Modified D'-D3 domain of VWF PRT

<400> 22

Glu Leu Tyr Cys Arg Pro Pro Met Val Lys Leu Val Cys Pro Ala Asp
 1 5 10 15

Asn Leu Arg Ala Glu Gly Leu Glu Cys Thr Lys Thr Cys Gln Asn Tyr
 20 25 30

Asp Leu Glu Cys Met Ser Met Gly Cys Val Ser Gly Cys Leu Cys Pro
 35 40 45

Pro Gly Met Val Arg His Glu Asn Arg Cys Val Ala Leu Glu Arg Cys
 50 55 60

Pro Cys Phe His Gln Gly Lys Glu Tyr Ala Pro Gly Glu Thr Val Lys
 65 70 75 80

Ile Gly Cys Asn Thr Cys Val Cys Arg Asp Arg Lys Trp Asn Cys Thr
85 90 95

Asp His Val Cys Asp Ala Thr Cys Ser Thr Ile Gly Met Ala His Tyr
100 105 110

Leu Thr Phe Asp Gly Leu Lys Tyr Leu Phe Pro Gly Glu Cys Gln Tyr
115 120 125

Val Leu Val Gln Asp Tyr Cys Gly Ser Asn Pro Gly Thr Phe Arg Ile
130 135 140

Leu Val Gly Asn Lys Gly Cys Ser His Pro Ser Val Lys Cys Lys Lys
145 150 155 160

Arg Val Thr Ile Leu Val Glu Gly Gly Glu Ile Glu Leu Phe Asp Gly
165 170 175

Glu Val Asn Val Lys Arg Pro Met Lys Asp Glu Thr His Phe Glu Val
180 185 190

Val Glu Ser Gly Arg Tyr Ile Ile Leu Leu Leu Gly Lys Ala Leu Ser
195 200 205

Val Val Trp Asp Arg His Leu Ser Ile Ser Val Val Leu Lys Gln Thr
210 215 220

Tyr Gln Glu Lys Val Cys Gly Leu Cys Gly Asn Phe Asp Gly Ile Gln
225 230 235 240

Asn Asn Asp Leu Thr Ser Ser Asn Leu Gln Val Glu Glu Asp Pro Val
245 250 255

Asp Phe Gly Asn Ser Trp Lys Val Ser Ser Gln Cys Ala Asp Thr Arg
260 265 270

Lys Val Pro Leu Asp Ser Ser Pro Ala Thr Cys His Asn Asn Ile Met
275 280 285

Lys Gln Thr Met Val Asp Ser Ser Cys Arg Ile Leu Thr Ser Asp Val
290 295 300

Phe Gln Asp Cys Asn Lys Leu Val Asp Pro Glu Pro Tyr Leu Asp Val
305 310 315 320

Cys Ile Tyr Asp Thr Cys Ser Cys Glu Ser Ile Gly Asp Cys Ala Cys
325 330 335

Phe Cys Asp Thr Ile Ala Ala Tyr Ala His Val Cys Ala Gln His Gly
340 345 350

Lys Val Val Thr Trp Arg Thr Ala Thr Leu Cys Pro Gln Ser Cys Glu
355 360 365

Glu Arg Asn Leu Arg Glu Asn Gly Tyr Glu Cys Glu Trp Arg Tyr Asn

370

375

380

Ser Cys Ala Pro Ala Cys Gln Val Thr Cys Gln His Pro Glu Pro Leu
385 390 395 400

Ala Cys Pro Val Gln Cys Val Glu Gly Cys His Ala His Cys Pro Pro
405 410 415

Gly Lys Ile Leu Asp Glu Leu Leu Gln Thr Cys Val Asp Pro Glu Asp
420 425 430

Cys Pro Val Cys Glu Val Ala Gly Arg Arg Phe Ala Ser Gly Lys Lys
435 440 445

Val Thr Leu Asn Pro Ser Asp Pro Glu His Cys Gln Ile Cys His Cys
450 455 460

Asp Val Val Asn Leu Thr Cys Glu Ala Cys Gln Glu Pro Gly Gly
465 470 475

<210> 23

<211> 479

<212> PRT

<213> Artificial sequence

<220>

<223> Modified D'-D3 domain of VWFPR

<400> 23

Tyr Leu Tyr Cys Arg Pro Pro Met Val Lys Leu Val Cys Pro Ala Asp
1 5 10 15

Asn Leu Arg Ala Glu Gly Leu Glu Cys Thr Lys Thr Cys Gln Asn Tyr
20 25 30

Asp Leu Glu Cys Met Ser Met Gly Cys Val Ser Gly Cys Leu Cys Pro
35 40 45

Pro Gly Met Val Arg His Glu Asn Arg Cys Val Ala Leu Glu Arg Cys
50 55 60

Pro Cys Phe His Gln Gly Lys Glu Tyr Ala Pro Gly Glu Thr Val Lys
65 70 75 80

Ile Gly Cys Asn Thr Cys Val Cys Arg Asp Arg Lys Trp Asn Cys Thr
85 90 95

Asp His Val Cys Asp Ala Thr Cys Ser Thr Ile Gly Met Ala His Tyr
100 105 110

Leu Thr Phe Asp Gly Leu Lys Tyr Leu Phe Pro Gly Glu Cys Gln Tyr
115 120 125

Val Leu Val Gln Asp Tyr Cys Gly Ser Asn Pro Gly Thr Phe Arg Ile

130 135 140
 Leu Val Gly Asn Lys Gly Cys Ser His Pro Ser Val Lys Cys Lys Lys
 145 150 155 160
 Arg Val Thr Ile Leu Val Glu Gly Gly Glu Ile Glu Leu Phe Asp Gly
 165 170 175
 Glu Val Asn Val Lys Arg Pro Met Lys Asp Glu Thr His Phe Glu Val
 180 185 190
 Val Glu Ser Gly Arg Tyr Ile Ile Leu Leu Leu Gly Lys Ala Leu Ser
 195 200 205
 Val Val Trp Asp Arg His Leu Ser Ile Ser Val Val Leu Lys Gln Thr
 210 215 220
 Tyr Gln Glu Lys Val Cys Gly Leu Cys Gly Asn Phe Asp Gly Ile Gln
 225 230 235 240
 Asn Asn Asp Leu Thr Ser Ser Asn Leu Gln Val Glu Glu Asp Pro Val
 245 250 255
 Asp Phe Gly Asn Ser Trp Lys Val Ser Ser Gln Cys Ala Asp Thr Arg
 260 265 270
 Lys Val Pro Leu Asp Ser Ser Pro Ala Thr Cys His Asn Asn Ile Met
 275 280 285
 Lys Gln Thr Met Val Asp Ser Ser Cys Arg Ile Leu Thr Ser Asp Val
 290 295 300
 Phe Gln Asp Cys Asn Lys Leu Val Asp Pro Glu Pro Tyr Leu Asp Val
 305 310 315 320
 Cys Ile Tyr Asp Thr Cys Ser Cys Glu Ser Ile Gly Asp Cys Ala Cys
 325 330 335
 Phe Cys Asp Thr Ile Ala Ala Tyr Ala His Val Cys Ala Gln His Gly
 340 345 350
 Lys Val Val Thr Trp Arg Thr Ala Thr Leu Cys Pro Gln Ser Cys Glu
 355 360 365
 Glu Arg Asn Leu Arg Glu Asn Gly Tyr Glu Cys Glu Trp Arg Tyr Asn
 370 375 380
 Ser Cys Ala Pro Ala Cys Gln Val Thr Cys Gln His Pro Glu Pro Leu
 385 390 395 400
 Ala Cys Pro Val Gln Cys Val Glu Gly Cys His Ala His Cys Pro Pro
 405 410 415
 Gly Lys Ile Leu Asp Glu Leu Leu Gln Thr Cys Val Asp Pro Glu Asp
 420 425 430

Cys Pro Val Cys Glu Val Ala Gly Arg Arg Phe Ala Ser Gly Lys Lys
 435 440 445

Val Thr Leu Asn Pro Ser Asp Pro Glu His Cys Gln Ile Cys His Cys
 450 455 460

Asp Val Val Asn Leu Thr Cys Glu Ala Cys Gln Glu Pro Gly Gly
 465 470 475

<210> 24

<211> 479

<212> PRT

<213> Artificial sequence

<220>

<223> Modified D'-D3 domain of VWFPR

<400> 24

Leu Leu Tyr Cys Arg Pro Pro Met Val Lys Leu Val Cys Pro Ala Asp
 1 5 10 15

Asn Leu Arg Ala Glu Gly Leu Glu Cys Thr Lys Thr Cys Gln Asn Tyr
 20 25 30

Asp Leu Glu Cys Met Ser Met Gly Cys Val Ser Gly Cys Leu Cys Pro
 35 40 45

Pro Gly Met Val Arg His Glu Asn Arg Cys Val Ala Leu Glu Arg Cys
 50 55 60

Pro Cys Phe His Gln Gly Lys Glu Tyr Ala Pro Gly Glu Thr Val Lys
 65 70 75 80

Ile Gly Cys Asn Thr Cys Val Cys Arg Asp Arg Lys Trp Asn Cys Thr
 85 90 95

Asp His Val Cys Asp Ala Thr Cys Ser Thr Ile Gly Met Ala His Tyr
 100 105 110

Leu Thr Phe Asp Gly Leu Lys Tyr Leu Phe Pro Gly Glu Cys Gln Tyr
 115 120 125

Val Leu Val Gln Asp Tyr Cys Gly Ser Asn Pro Gly Thr Phe Arg Ile
 130 135 140

Leu Val Gly Asn Lys Gly Cys Ser His Pro Ser Val Lys Cys Lys Lys
 145 150 155 160

Arg Val Thr Ile Leu Val Glu Gly Gly Glu Ile Glu Leu Phe Asp Gly
 165 170 175

Glu Val Asn Val Lys Arg Pro Met Lys Asp Glu Thr His Phe Glu Val
 180 185 190

Val Glu Ser Gly Arg Tyr Ile Ile Leu Leu Leu Gly Lys Ala Leu Ser
 195 200 205

Val Val Trp Asp Arg His Leu Ser Ile Ser Val Val Leu Lys Gln Thr
 210 215 220

Tyr Gln Glu Lys Val Cys Gly Leu Cys Gly Asn Phe Asp Gly Ile Gln
 225 230 235 240

Asn Asn Asp Leu Thr Ser Ser Asn Leu Gln Val Glu Glu Asp Pro Val
 245 250 255

Asp Phe Gly Asn Ser Trp Lys Val Ser Ser Gln Cys Ala Asp Thr Arg
 260 265 270

Lys Val Pro Leu Asp Ser Ser Pro Ala Thr Cys His Asn Asn Ile Met
 275 280 285

Lys Gln Thr Met Val Asp Ser Ser Cys Arg Ile Leu Thr Ser Asp Val
 290 295 300

Phe Gln Asp Cys Asn Lys Leu Val Asp Pro Glu Pro Tyr Leu Asp Val
 305 310 315 320

Cys Ile Tyr Asp Thr Cys Ser Cys Glu Ser Ile Gly Asp Cys Ala Cys
 325 330 335

Phe Cys Asp Thr Ile Ala Ala Tyr Ala His Val Cys Ala Gln His Gly
 340 345 350

Lys Val Val Thr Trp Arg Thr Ala Thr Leu Cys Pro Gln Ser Cys Glu
 355 360 365

Glu Arg Asn Leu Arg Glu Asn Gly Tyr Glu Cys Glu Trp Arg Tyr Asn
 370 375 380

Ser Cys Ala Pro Ala Cys Gln Val Thr Cys Gln His Pro Glu Pro Leu
 385 390 395 400

Ala Cys Pro Val Gln Cys Val Glu Gly Cys His Ala His Cys Pro Pro
 405 410 415

Gly Lys Ile Leu Asp Glu Leu Leu Gln Thr Cys Val Asp Pro Glu Asp
 420 425 430

Cys Pro Val Cys Glu Val Ala Gly Arg Arg Phe Ala Ser Gly Lys Lys
 435 440 445

Val Thr Leu Asn Pro Ser Asp Pro Glu His Cys Gln Ile Cys His Cys
 450 455 460

Asp Val Val Asn Leu Thr Cys Glu Ala Cys Gln Glu Pro Gly Gly
 465 470 475

<210> 25

<211> 479

<212> PRT

<213> Artificial sequence

<220>

<223> Modified D'-D3 domain of VWFPR

<400> 25

Pro Leu Trp Cys Arg Pro Pro Met Val Lys Leu Val Cys Pro Ala Asp
 1 5 10 15

Asn Leu Arg Ala Glu Gly Leu Glu Cys Thr Lys Thr Cys Gln Asn Tyr
 20 25 30

Asp Leu Glu Cys Met Ser Met Gly Cys Val Ser Gly Cys Leu Cys Pro
 35 40 45

Pro Gly Met Val Arg His Glu Asn Arg Cys Val Ala Leu Glu Arg Cys
 50 55 60

Pro Cys Phe His Gln Gly Lys Glu Tyr Ala Pro Gly Glu Thr Val Lys
 65 70 75 80

Ile Gly Cys Asn Thr Cys Val Cys Arg Asp Arg Lys Trp Asn Cys Thr
 85 90 95

Asp His Val Cys Asp Ala Thr Cys Ser Thr Ile Gly Met Ala His Tyr
 100 105 110

Leu Thr Phe Asp Gly Leu Lys Tyr Leu Phe Pro Gly Glu Cys Gln Tyr
 115 120 125

Val Leu Val Gln Asp Tyr Cys Gly Ser Asn Pro Gly Thr Phe Arg Ile
 130 135 140

Leu Val Gly Asn Lys Gly Cys Ser His Pro Ser Val Lys Cys Lys Lys
 145 150 155 160

Arg Val Thr Ile Leu Val Glu Gly Gly Glu Ile Glu Leu Phe Asp Gly
 165 170 175

Glu Val Asn Val Lys Arg Pro Met Lys Asp Glu Thr His Phe Glu Val
 180 185 190

Val Glu Ser Gly Arg Tyr Ile Ile Leu Leu Leu Gly Lys Ala Leu Ser
 195 200 205

Val Val Trp Asp Arg His Leu Ser Ile Ser Val Val Leu Lys Gln Thr
 210 215 220

Tyr Gln Glu Lys Val Cys Gly Leu Cys Gly Asn Phe Asp Gly Ile Gln
 225 230 235 240

Asn Asn Asp Leu Thr Ser Ser Asn Leu Gln Val Glu Glu Asp Pro Val

245	250	255
Asp Phe Gly Asn Ser Trp Lys Val Ser Ser Gln Cys Ala Asp Thr Arg		
260	265	270
Lys Val Pro Leu Asp Ser Ser Pro Ala Thr Cys His Asn Asn Ile Met		
275	280	285
Lys Gln Thr Met Val Asp Ser Ser Cys Arg Ile Leu Thr Ser Asp Val		
290	295	300
Phe Gln Asp Cys Asn Lys Leu Val Asp Pro Glu Pro Tyr Leu Asp Val		
305	310	315
Cys Ile Tyr Asp Thr Cys Ser Cys Glu Ser Ile Gly Asp Cys Ala Cys		
325	330	335
Phe Cys Asp Thr Ile Ala Ala Tyr Ala His Val Cys Ala Gln His Gly		
340	345	350
Lys Val Val Thr Trp Arg Thr Ala Thr Leu Cys Pro Gln Ser Cys Glu		
355	360	365
Glu Arg Asn Leu Arg Glu Asn Gly Tyr Glu Cys Glu Trp Arg Tyr Asn		
370	375	380
Ser Cys Ala Pro Ala Cys Gln Val Thr Cys Gln His Pro Glu Pro Leu		
385	390	395
Ala Cys Pro Val Gln Cys Val Glu Gly Cys His Ala His Cys Pro Pro		
405	410	415
Gly Lys Ile Leu Asp Glu Leu Leu Gln Thr Cys Val Asp Pro Glu Asp		
420	425	430
Cys Pro Val Cys Glu Val Ala Gly Arg Arg Phe Ala Ser Gly Lys Lys		
435	440	445
Val Thr Leu Asn Pro Ser Asp Pro Glu His Cys Gln Ile Cys His Cys		
450	455	460
Asp Val Val Asn Leu Thr Cys Glu Ala Cys Gln Glu Pro Gly Gly		
465	470	475
<210> 26		
<211> 479		
<212> PRT		
<213> Artificial sequence		
<220>		
<223> Modified D'-D3 domain of VWFPR		
<400> 26		
Ser Leu Thr Cys Arg Pro Pro Met Val Lys Leu Val Cys Pro Ala Asp		

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Ser Leu Asp Cys Arg Phe Phe Met Val Lys Leu Val Cys Phe Ala Asp
1          5          10          15

Asn Leu Arg Ala Glu Gly Leu Glu Cys Thr Lys Thr Cys Gln Asn Tyr
20          25          30

Asp Leu Glu Cys Met Ser Met Gly Cys Val Ala Gly Cys Leu Cys Pro
35          40          45

Pro Gly Met Val Arg His Glu Asn Arg Cys Val Ala Leu Glu Arg Cys
50          55          60

Pro Cys Phe His Gln Gly Lys Glu Tyr Ala Pro Gly Glu Thr Val Lys
65          70          75          80

Ile Gly Cys Asn Thr Cys Val Cys Arg Asp Arg Lys Trp Asn Cys Thr
85          90          95

Asp His Val Cys Asp Ala Thr Cys Ser Thr Ile Gly Met Ala His Tyr
100         105         110

Leu Thr Phe Asp Gly Leu Lys Tyr Leu Phe Pro Gly Glu Cys Gln Tyr
115         120         125

Val Leu Val Gln Asp Tyr Cys Gly Ser Asn Pro Gly Thr Phe Arg Ile
130         135         140

Leu Val Gly Asn Lys Gly Cys Ser His Pro Ser Val Lys Cys Lys Lys
145         150         155         160

Arg Val Thr Ile Leu Val Glu Gly Gly Glu Ile Glu Leu Phe Asp Gly
165         170         175

Glu Val Asn Val Lys Arg Pro Met Lys Asp Glu Thr His Phe Glu Val
180         185         190

Val Glu Ser Gly Arg Tyr Ile Ile Leu Leu Leu Gly Lys Ala Leu Ser
195         200         205

Val Val Trp Asp Arg His Leu Ser Ile Ser Val Val Leu Lys Gln Thr
210         215         220

Tyr Gln Glu Lys Val Cys Gly Leu Cys Gly Asn Phe Asp Gly Ile Gln
225         230         235         240

Asn Asn Asp Leu Thr Ser Ser Asn Leu Gln Val Glu Glu Asp Pro Val
245         250         255

Asp Phe Gly Asn Ser Trp Lys Val Ser Ser Gln Cys Ala Asp Thr Arg
260         265         270

Lys Val Pro Leu Asp Ser Ser Pro Ala Thr Cys His Asn Asn Ile Met
275         280         285

Lys Gln Thr Met Val Asp Ser Ser Cys Arg Ile Leu Thr Ser Asp Val
290         295         300

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Phe Gln Asp Cys Asn Lys Leu Val Asp Pro Glu Pro Tyr Leu Asp Val

305 310 315 320

Cys Ile Tyr Asp Thr Cys Ser Cys Glu Ser Ile Gly Asp Cys Ala Cys
 325 330 335

Phe Cys Asp Thr Ile Ala Ala Tyr Ala His Val Cys Ala Gln His Gly
 340 345 350

Lys Val Val Thr Trp Arg Thr Ala Thr Leu Cys Pro Gln Ser Cys Glu
 355 360 365

Glu Arg Asn Leu Arg Glu Asn Gly Tyr Glu Cys Glu Trp Arg Tyr Asn
 370 375 380

Ser Cys Ala Pro Ala Cys Gln Val Thr Cys Gln His Pro Glu Pro Leu
385 390 395 400

Ala Cys Pro Val Gln Cys Val Glu Gly Cys His Ala His Cys Pro Pro
 405 410 415

Gly Lys Ile Leu Asp Glu Leu Leu Gln Thr Cys Val Asp Pro Glu Asp
 420 425 430

Cys Pro Val Cys Glu Val Ala Gly Arg Arg Phe Ala Ser Gly Lys Lys
 435 440 445

Val Thr Leu Asn Pro Ser Asp Pro Glu His Cys Gln Ile Cys His Cys
 450 455 460

Asp Val Val Asn Leu Thr Cys Glu Ala Cys Gln Glu Pro Gly Gly
465 470 475

<210> 27

<211> 479

<212> PRT

<213> Artificial sequence

<220>

<223> Modified D'-D3 domain of VWFPR

<400> 27

Ser Leu Tyr Cys Arg Lys Pro Met Val Lys Leu Val Cys Pro Ala Asp
1 5 10 15

Asn Leu Arg Ala Glu Gly Leu Glu Cys Thr Lys Thr Cys Gln Asn Tyr
 20 25 30

Asp Leu Glu Cys Met Ser Met Gly Cys Val Ser Gly Cys Leu Cys Pro
 35 40 45

Pro Gly Met Val Arg His Glu Asn Arg Cys Val Ala Leu Glu Arg Cys
50 55 60

Pro Cys Phe His Gln Gly Lys Glu Tyr Ala Pro Gly Glu Thr Val Lys
 65 70 75 80

Ile Gly Cys Asn Thr Cys Val Cys Arg Asp Arg Lys Trp Asn Cys Thr
 85 90 95

Asp His Val Cys Asp Ala Thr Cys Ser Thr Ile Gly Met Ala His Tyr
 100 105 110

Leu Thr Phe Asp Gly Leu Lys Tyr Leu Phe Pro Gly Glu Cys Gln Tyr
 115 120 125

Val Leu Val Gln Asp Tyr Cys Gly Ser Asn Pro Gly Thr Phe Arg Ile
 130 135 140

Leu Val Gly Asn Lys Gly Cys Ser His Pro Ser Val Lys Cys Lys Lys
 145 150 155 160

Arg Val Thr Ile Leu Val Glu Gly Gly Glu Ile Glu Leu Phe Asp Gly
 165 170 175

Glu Val Asn Val Lys Arg Pro Met Lys Asp Glu Thr His Phe Glu Val
 180 185 190

Val Glu Ser Gly Arg Tyr Ile Ile Leu Leu Leu Gly Lys Ala Leu Ser
 195 200 205

Val Val Trp Asp Arg His Leu Ser Ile Ser Val Val Leu Lys Gln Thr
 210 215 220

Tyr Gln Glu Lys Val Cys Gly Leu Cys Gly Asn Phe Asp Gly Ile Gln
 225 230 235 240

Asn Asn Asp Leu Thr Ser Ser Asn Leu Gln Val Glu Glu Asp Pro Val
 245 250 255

Asp Phe Gly Asn Ser Trp Lys Val Ser Ser Gln Cys Ala Asp Thr Arg
 260 265 270

Lys Val Pro Leu Asp Ser Ser Pro Ala Thr Cys His Asn Asn Ile Met
 275 280 285

Lys Gln Thr Met Val Asp Ser Ser Cys Arg Ile Leu Thr Ser Asp Val
 290 295 300

Phe Gln Asp Cys Asn Lys Leu Val Asp Pro Glu Pro Tyr Leu Asp Val
 305 310 315 320

Cys Ile Tyr Asp Thr Cys Ser Cys Glu Ser Ile Gly Asp Cys Ala Cys
 325 330 335

Phe Cys Asp Thr Ile Ala Ala Tyr Ala His Val Cys Ala Gln His Gly
 340 345 350

Lys Val Val Thr Trp Arg Thr Ala Thr Leu Cys Pro Gln Ser Cys Glu
355 360 365

Glu Arg Asn Leu Arg Glu Asn Gly Tyr Glu Cys Glu Trp Arg Tyr Asn
370 375 380

Ser Cys Ala Pro Ala Cys Gln Val Thr Cys Gln His Pro Glu Pro Leu
385 390 395 400

Ala Cys Pro Val Gln Cys Val Glu Gly Cys His Ala His Cys Pro Pro
405 410 415

Gly Lys Ile Leu Asp Glu Leu Leu Gln Thr Cys Val Asp Pro Glu Asp
420 425 430

Cys Pro Val Cys Glu Val Ala Gly Arg Arg Phe Ala Ser Gly Lys Lys
435 440 445

Val Thr Leu Asn Pro Ser Asp Pro Glu His Cys Gln Ile Cys His Cys
450 455 460

Asp Val Val Asn Leu Thr Cys Glu Ala Cys Gln Glu Pro Gly Gly
465 470 475

<210> 28

<211> 479

<212> PRT

<213> Artificial sequence

<220>

<223> Modified D'-D3 domain of VWFPR

<400> 28

Ser Leu Tyr Cys Arg Asn Pro Met Val Lys Leu Val Cys Pro Ala Asp
1 5 10 15

Asn Leu Arg Ala Glu Gly Leu Glu Cys Thr Lys Thr Cys Gln Asn Tyr
20 25 30

Asp Leu Glu Cys Met Ser Met Gly Cys Val Ser Gly Cys Leu Cys Pro
35 40 45

Pro Gly Met Val Arg His Glu Asn Arg Cys Val Ala Leu Glu Arg Cys
50 55 60

Pro Cys Phe His Gln Gly Lys Glu Tyr Ala Pro Gly Glu Thr Val Lys
65 70 75 80

Ile Gly Cys Asn Thr Cys Val Cys Arg Asp Arg Lys Trp Asn Cys Thr
85 90 95

Asp His Val Cys Asp Ala Thr Cys Ser Thr Ile Gly Met Ala His Tyr
100 105 110

Leu Thr Phe Asp Gly Leu Lys Tyr Leu Phe Pro Gly Glu Cys Gln Tyr
115 120 125

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Val Leu Val Gln Asp Tyr Cys Gly Ser Asn Pro Gly Thr Phe Arg Ile
130                      135                      140

Leu Val Gly Asn Lys Gly Cys Ser His Pro Ser Val Lys Cys Lys Lys
145                      150                      155                      160

Arg Val Thr Ile Leu Val Glu Gly Gly Glu Ile Glu Leu Phe Asp Gly
165                      170                      175

Glu Val Asn Val Lys Arg Pro Met Lys Asp Glu Thr His Phe Glu Val
180                      185                      190

Val Glu Ser Gly Arg Tyr Ile Ile Leu Leu Leu Gly Lys Ala Leu Ser
195                      200                      205

Val Val Trp Asp Arg His Leu Ser Ile Ser Val Val Leu Lys Gln Thr
210                      215                      220

Tyr Gln Glu Lys Val Cys Gly Leu Cys Gly Asn Phe Asp Gly Ile Gln
225                      230                      235                      240

Asn Asn Asp Leu Thr Ser Ser Asn Leu Gln Val Glu Glu Asp Pro Val
245                      250                      255

Asp Phe Gly Asn Ser Trp Lys Val Ser Ser Gln Cys Ala Asp Thr Arg
260                      265                      270

Lys Val Pro Leu Asp Ser Ser Pro Ala Thr Cys His Asn Asn Ile Met
275                      280                      285

Lys Gln Thr Met Val Asp Ser Ser Cys Arg Ile Leu Thr Ser Asp Val
290                      295                      300

Phe Gln Asp Cys Asn Lys Leu Val Asp Pro Glu Pro Tyr Leu Asp Val
305                      310                      315                      320

Cys Ile Tyr Asp Thr Cys Ser Cys Glu Ser Ile Gly Asp Cys Ala Cys
325                      330                      335

Phe Cys Asp Thr Ile Ala Ala Tyr Ala His Val Cys Ala Gln His Gly
340                      345                      350

Lys Val Val Thr Trp Arg Thr Ala Thr Leu Cys Pro Gln Ser Cys Glu
355                      360                      365

Glu Arg Asn Leu Arg Glu Asn Gly Tyr Glu Cys Glu Trp Arg Tyr Asn
370                      375                      380

Ser Cys Ala Pro Ala Cys Gln Val Thr Cys Gln His Pro Glu Pro Leu
385                      390                      395                      400

Ala Cys Pro Val Gln Cys Val Glu Gly Cys His Ala His Cys Pro Pro
405                      410                      415

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Gly Lys Ile Leu Asp Glu Leu Leu Gln Thr Cys Val Asp Pro Glu Asp
 420 425 430

Cys Pro Val Cys Glu Val Ala Gly Arg Arg Phe Ala Ser Gly Lys Lys
 435 440 445

Val Thr Leu Asn Pro Ser Asp Pro Glu His Cys Gln Ile Cys His Cys
 450 455 460

Asp Val Val Asn Leu Thr Cys Glu Ala Cys Gln Glu Pro Gly Gly
 465 470 475

<210> 29

<211> 479

<212> PRT

<213> Artificial sequence

<220>

<223> Modified D'-D3 domain of VWFPR

<400> 29

Ser Leu Tyr Cys Arg Arg Pro Met Val Lys Leu Val Cys Pro Ala Asp
 1 5 10 15

Asn Leu Arg Ala Glu Gly Leu Glu Cys Thr Lys Thr Cys Gln Asn Tyr
 20 25 30

Asp Leu Glu Cys Met Ser Met Gly Cys Val Ser Gly Cys Leu Cys Pro
 35 40 45

Pro Gly Met Val Arg His Glu Asn Arg Cys Val Ala Leu Glu Arg Cys
 50 55 60

Pro Cys Phe His Gln Gly Lys Glu Tyr Ala Pro Gly Glu Thr Val Lys
 65 70 75 80

Ile Gly Cys Asn Thr Cys Val Cys Arg Asp Arg Lys Trp Asn Cys Thr
 85 90 95

Asp His Val Cys Asp Ala Thr Cys Ser Thr Ile Gly Met Ala His Tyr
 100 105 110

Leu Thr Phe Asp Gly Leu Lys Tyr Leu Phe Pro Gly Glu Cys Gln Tyr
 115 120 125

Val Leu Val Gln Asp Tyr Cys Gly Ser Asn Pro Gly Thr Phe Arg Ile
 130 135 140

Leu Val Gly Asn Lys Gly Cys Ser His Pro Ser Val Lys Cys Lys Lys
 145 150 155 160

Arg Val Thr Ile Leu Val Glu Gly Gly Glu Ile Glu Leu Phe Asp Gly
 165 170 175

Glu Val Asn Val Lys Arg Pro Met Lys Asp Glu Thr His Phe Glu Val
 180 185 190

Val Glu Ser Gly Arg Tyr Ile Ile Leu Leu Leu Gly Lys Ala Leu Ser
 195 200 205

Val Val Trp Asp Arg His Leu Ser Ile Ser Val Val Leu Lys Gln Thr
 210 215 220

Tyr Gln Glu Lys Val Cys Gly Leu Cys Gly Asn Phe Asp Gly Ile Gln
 225 230 235 240

Asn Asn Asp Leu Thr Ser Ser Asn Leu Gln Val Glu Glu Asp Pro Val
 245 250 255

Asp Phe Gly Asn Ser Trp Lys Val Ser Ser Gln Cys Ala Asp Thr Arg
 260 265 270

Lys Val Pro Leu Asp Ser Ser Pro Ala Thr Cys His Asn Asn Ile Met
 275 280 285

Lys Gln Thr Met Val Asp Ser Ser Cys Arg Ile Leu Thr Ser Asp Val
 290 295 300

Phe Gln Asp Cys Asn Lys Leu Val Asp Pro Glu Pro Tyr Leu Asp Val
 305 310 315 320

Cys Ile Tyr Asp Thr Cys Ser Cys Glu Ser Ile Gly Asp Cys Ala Cys
 325 330 335

Phe Cys Asp Thr Ile Ala Ala Tyr Ala His Val Cys Ala Gln His Gly
 340 345 350

Lys Val Val Thr Trp Arg Thr Ala Thr Leu Cys Pro Gln Ser Cys Glu
 355 360 365

Glu Arg Asn Leu Arg Glu Asn Gly Tyr Glu Cys Glu Trp Arg Tyr Asn
 370 375 380

Ser Cys Ala Pro Ala Cys Gln Val Thr Cys Gln His Pro Glu Pro Leu
 385 390 395 400

Ala Cys Pro Val Gln Cys Val Glu Gly Cys His Ala His Cys Pro Pro
 405 410 415

Gly Lys Ile Leu Asp Glu Leu Leu Gln Thr Cys Val Asp Pro Glu Asp
 420 425 430

Cys Pro Val Cys Glu Val Ala Gly Arg Arg Phe Ala Ser Gly Lys Lys
 435 440 445

Val Thr Leu Asn Pro Ser Asp Pro Glu His Cys Gln Ile Cys His Cys
 450 455 460

Asp Val Val Asn Leu Thr Cys Glu Ala Cys Gln Glu Pro Gly Gly
 465 470 475

<210> 30

<211> 479

<212> PRT

<213> Artificial sequence

<220>

<223> Modified D'-D3 domain of VWFPRT

<400> 30

Pro Leu Leu Cys Arg Pro Pro Met Val Lys Leu Val Cys Pro Ala Asp
 1 5 10 15

Asn Leu Arg Ala Glu Gly Leu Glu Cys Thr Lys Thr Cys Gln Asn Tyr
 20 25 30

Asp Leu Glu Cys Met Ser Met Gly Cys Val Ser Gly Cys Leu Cys Pro
 35 40 45

Pro Gly Met Val Arg His Glu Asn Arg Cys Val Ala Leu Glu Arg Cys
 50 55 60

Pro Cys Phe His Gln Gly Lys Glu Tyr Ala Pro Gly Glu Thr Val Lys
 65 70 75 80

Ile Gly Cys Asn Thr Cys Val Cys Arg Asp Arg Lys Trp Asn Cys Thr
 85 90 95

Asp His Val Cys Asp Ala Thr Cys Ser Thr Ile Gly Met Ala His Tyr
 100 105 110

Leu Thr Phe Asp Gly Leu Lys Tyr Leu Phe Pro Gly Glu Cys Gln Tyr
 115 120 125

Val Leu Val Gln Asp Tyr Cys Gly Ser Asn Pro Gly Thr Phe Arg Ile
 130 135 140

Leu Val Gly Asn Lys Gly Cys Ser His Pro Ser Val Lys Cys Lys Lys
 145 150 155 160

Arg Val Thr Ile Leu Val Glu Gly Gly Glu Ile Glu Leu Phe Asp Gly
 165 170 175

Glu Val Asn Val Lys Arg Pro Met Lys Asp Glu Thr His Phe Glu Val
 180 185 190

Val Glu Ser Gly Arg Tyr Ile Ile Leu Leu Leu Gly Lys Ala Leu Ser
 195 200 205

Val Val Trp Asp Arg His Leu Ser Ile Ser Val Val Leu Lys Gln Thr
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PATENTKRAV

1. Et polypeptid omfattende trunkeret von Willebrand Faktor (VWF), som omfatter en sekvens som vist i SEQ ID NO:3 eller et fragment deraf, hvor den trunkerede VWF
5 omfatter SEQ ID NO:5 (S764P/S766W/V1083A), SEQ ID NO:5 ID NO:6 (S764G/S766Y/V1083A), SEQ ID NO:7 (S764E/S766Y/V1083A), SEQ ID NO:8 (N1011S/V1083A/K1181E), SEQ ID NO:17 (S766Y/V1083), SEQ ID NO:8 ID NO:9 (V1083A), SEQ ID NO:10 (S1042T), SEQ ID NO:11 (V805A/Q1158L), SEQ ID NO:12 (K912E/T1088S) eller SEQ ID NO:13 (L781P); og hvor den trunkerede VWF
10 binder Faktor VIII (FVIII), og den trunkerede VWF binder til Faktor VIII med en off-rate, der er lavere end et referencepolypeptid, der omfatter et umodificeret SEQ ID NO:3.
2. Polypeptid ifølge krav 1, hvor det trunkerede VWF binder til faktor VIII med en off-rate, der er mindst 5 gange lavere end referencepolypeptidet; hvorved den trunkerede VWF binder til faktor VIII foretrukket med en off-rate, der er mindst 10 gange lavere end referencepolypeptidet.
3. Polypeptid ifølge krav 1, hvor det trunkerede VWF binder til faktor VIII med en KD,
20 der er mindst 5 gange lavere end referencepolypeptidet; hvorved den trunkerede VWF binder til faktor VIII, foretrukket med en KD, der er mindst 10 gange lavere end referencepolypeptidet.
4. Polypeptid ifølge et hvilket som helst af kravene 1 til 3, hvor den trunkerede VWF
25 yderligere omfatter resterne 1243 til 1247 af SEQ ID NO:2 eller yderligere omfatter resterne 1243 til 1270 af SEQ ID NO:2.
5. Polypeptid ifølge et hvilket som helst af kravene 1 til 3, hvor den trunkerede VWF mangler resterne 1243 til 1247 af SEQ ID NO:2 eller mangler resterne 1243 til 2813 af
30 SEQ ID NO:2.
6. Polypeptid ifølge et hvilket som helst af kravene 1 til 5, hvor polypeptidet yderligere omfatter en halveringstidsforlængende del.

7. Polypeptid ifølge krav 6, hvor den halveringstidsforlængende del er en heterolog aminosyresekvens fusioneret til den trunke-
rede VWF, hvor den heterologe aminosyre-
5 sekvens foretrukket omfatter eller består af et polypeptid udvalgt blandt gruppen bestå-
ende af immunoglobulin konstante områder og dele deraf, f.eks Fc-fragmentet, trans-
ferrin og fragmenter deraf, det C-terminale peptid af humant choriongonadotropin, sol-
vatiserede tilfældige kæder med stort hydrodynamisk volumen kendt som XTEN,
homo-aminosyregentagelser (HAP), prolin-alanin-serin-gentagelser (PAS), albumin,
afamin, alfa-fetoprotein, vitamin D bindende protein, polypeptider, der er i stand til at
10 binde under fysiologiske betingelser til albumin eller immunoglobulin konstante regio-
ner, og kombinationer deraf.

8. Polypeptid ifølge krav 6, hvor den halveringstidsforlængende del er konjugeret til
polypeptidet, hvor den halveringstidsforlængende del foretrukket er udvalgt blandt
15 gruppen bestående af hydroxyethylstivelse (HES), polyethylenglycol (PEG), polysial-
insyrer (PSA), elastinlignende polypeptider, heparosanpolymerer, hyaluronsyre og al-
buminbindende ligander, f.eks. fedtsyrekæder og kombinationer deraf.

9. Polypeptid ifølge krav 7, hvor den heterologe aminosyresekvens omfatter albumin,
20 foretrukket, hvor N-terminalen af albumin er fusioneret til C-terminalen af den trunke-
rede VWF-sekvens enten direkte eller via en spacer.

10. Polypeptid ifølge et af kravene 1 til 9, hvor polypeptidet er et glycoprotein omfat-
tende N-glycaner, og hvor mindst 75%, foretrukket mindst 85%, foretrukket mindst
25 90%, og mere foretrukket mindst 95%. af N-glycanerne omfatter i gennemsnit mindst
én sialinsyredel, foretrukket hvor mindst 60% af N-glycanerne i gennemsnit omfatter
mindst én a-2,6-sialinsyredel.

11. Polypeptid ifølge et hvilket som helst af kravene 1 til 10, hvor polypeptidet er en
30 dimer.

12. Kompleks omfattende et faktor VIII-molekyle og polypeptidet ifølge et hvilket som
helst af kravene 1 til 11.

13. Polypeptid ifølge et hvilket som helst af kravene 1 til 11 eller komplekset ifølge krav 12 til anvendelse i behandling eller profylakse af en blodkoagulationsforstyrrelse, hvor blodkoagulationsforstyrrelsen foretrukket er von Willebrands sygdom (VWD) eller hæmofili A.

5

14. Farmaceutisk sammensætning omfattende polypeptidet ifølge et hvilket som helst af kravene 1 til 11 eller komplekset ifølge krav 12.

15. Farmaceutisk sammensætning omfattende (i) en FVIII og (ii) et polypeptid ifølge et hvilket som helst af kravene 1 til 11, hvor molforholdet mellem polypeptidet og FVIII i sammensætningen er større end 50.

16. Farmaceutisk kit omfattende (i) en FVIII og (ii) et polypeptid som defineret i et hvilket som helst af kravene 1 til 11 til samtidig, separat eller sekventiel anvendelse i behandlingen af en blodkoagulationsforstyrrelse, hvilken behandling omfatter administration til et individ med endogen VWF polypeptidet og FVIII, hvor molforholdet mellem polypeptidet administreret til det endogene VWF er større end 0,5, og/eller hvor molforholdet mellem polypeptidet, der skal administreres til FVIII, der skal administreres, er større end 50.

20

17. Polypeptid til anvendelse som defineret i krav 13 til forbedring af plasmahalveringstiden af FVIII og/eller til reducere hyppigheden af administration af FVIII.

18. Polynukleotid, der koder for polypeptidet ifølge et hvilket som helst af kravene 1 til 11.

25

19. Plasmid eller vektor omfattende polynukleotidet ifølge krav 18, hvor plasmidet eller vektoren foretrukket er en ekspressionsvektor.

20. Værtscelle omfattende polynukleotidet ifølge krav 18 eller plasmidet ifølge krav 19.

30

21. Fremgangsmåde til fremstilling af et polypeptid omfattende en trunkeret VWF, omfattende:

- (i) dyrkning af værtscellerne ifølge krav 20 under sådanne betingelser, at polypeptidet, der omfatter en trunkeret VWF, udtrykkes; og
- (ii) eventuelt udvinding af polypeptidet omfattende den trunkerede VWF fra værtscellerne eller fra dyrkningsmediet.

DRAWINGS

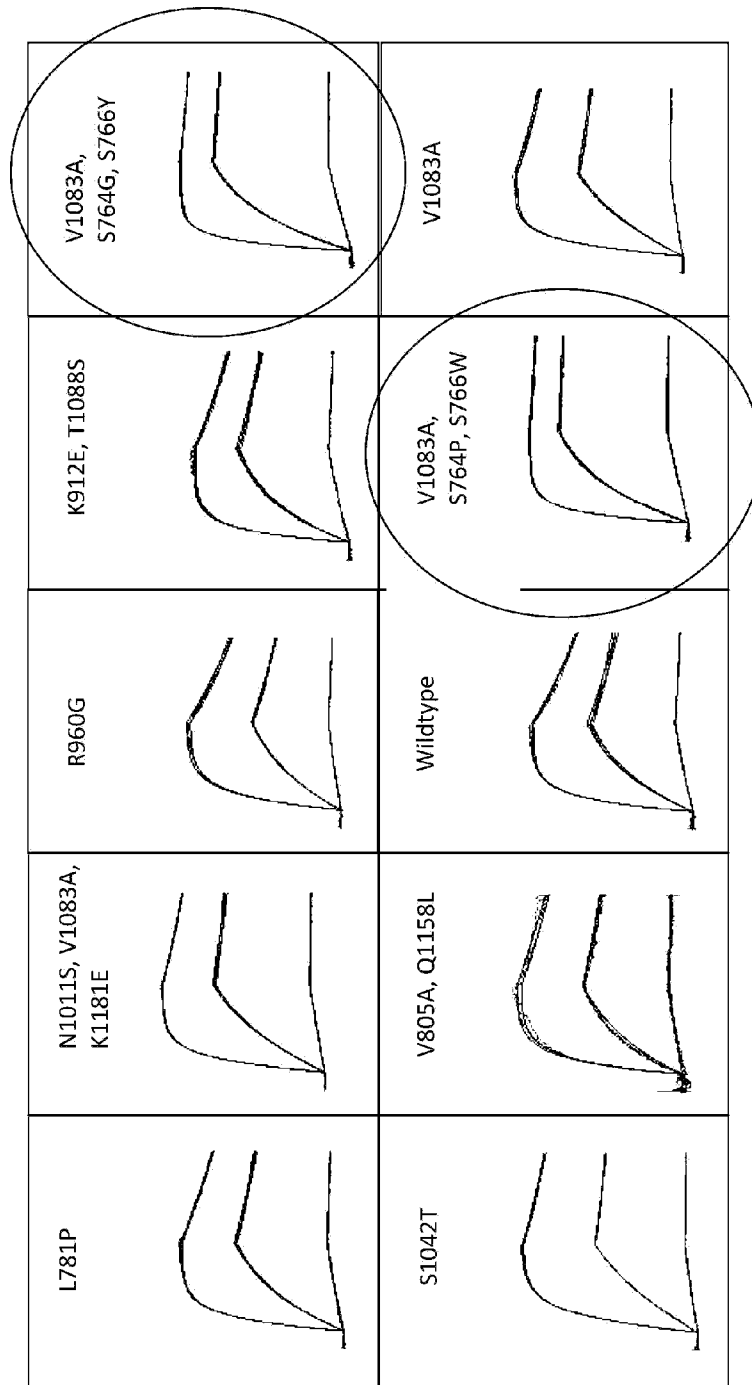


Figure 1

Figure 2

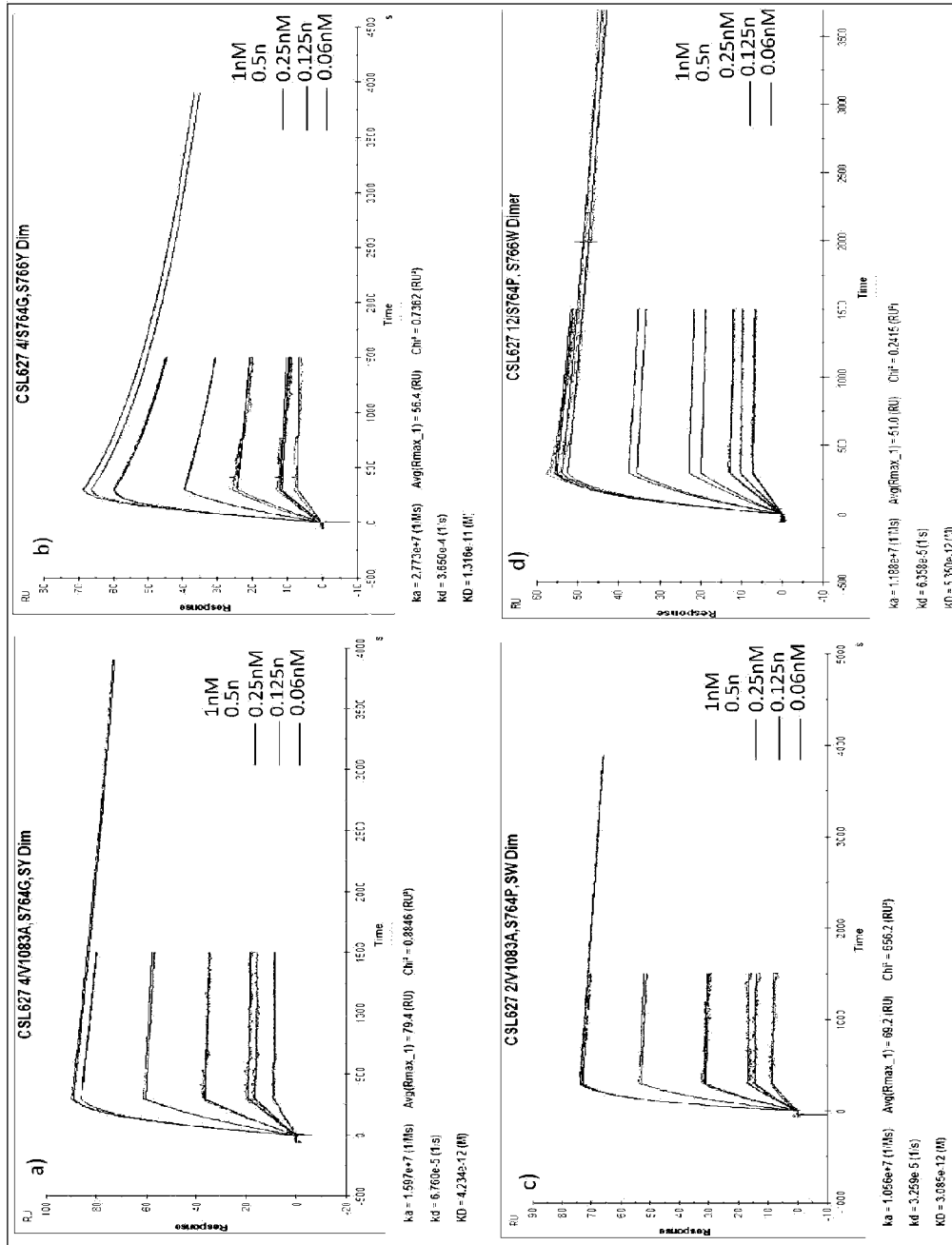


Figure 3

