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DESCRIPTION

BACKGROUND

[0001] Platelet activation and subsequent thrombus formation at sites of vascular injury is crucial for normal hemostasis, but it can also cause myocardial infarction and stroke (Coughlin SR. Nature. 2000; 407: 258-64). Platelet adhesion and activation is a multistep process involving multiple platelet receptor-ligand interactions. Upon vessel wall injury, circulating platelets are rapidly decelerated by transient interactions of the glycoprotein (GP) Ib-V-IX complex with von Willebrand factor (vWF) immobilized on the exposed subendothelial extracellular matrix (e.g. on collagen) (Shapiro MJ, et al. The Journal of Biological Chemistry. 2000; 275: 25216-21). This interaction retains platelets close to the vessel wall and facilitates the contact between GPVI and collagen (Nieswandt B, et al. Blood. 2003; 102: 449-61). GPVI-collagen interactions induce an intracellular signaling cascade leading to platelet activation and the release of secondary platelet agonists, such as thromboxane A_2 (TxA_2) and adenosine diphosphate (ADP). These soluble agonists together with locally produced thrombin further contribute to platelet activation through G protein (G_i, G_q, $G_{12/13}$) coupled receptors (Offermanns S. Circulation research. 2006; 99: 1293-304). All these signaling pathways synergize to induce complex cellular responses, such as activation of integrins, release of granule contents and the provision of a pro-coagulant surface for the activation of the coagulation cascade (Nakanishi-Matsui M, et al. Nature. 2000; 404: 609-13; Cunningham MA, et al. The Journal of experimental medicine. 2000; 191: 455-62). The final thrombus is embedded in a fibrin network to withstand the shear forces generated by the flowing blood. The stabilization of a newly formed thrombus is essential to arrest bleeding at sites of vascular injury. However, if this process occurs in an uncontrolled manner it may also lead to thrombotic events causing life-threatening disease states such as myocardial infarction or ischemic stroke. Consequently, antiplatelet and anticoagulant drugs, used alone or in combination, are of major importance in treating cardio- and cerebrovascular diseases (May F, et al. Blood. 2009; 114: 3464-72; Schroder J, et al. Mol Cell Biol. 2009; 29: 1083-94; Braun A, et al. Blood. 2009; 113: 2056-63). Whilst current anti-platelet therapies reduce the recurrence of vascular events, the increased risk of bleeding due to platelet inhibition is a particular concern for patients who have experienced stroke, and a further subset of patients remain refractory to anti-platelet approaches (Pleines I, et al. Pflugers Archiv : European journal of physiology. 2009; 457: 1173-85), underscoring the need for novel anti-platelet strategies.

[0002] Their central role in platelet adhesion puts two receptor complexes in the focus of platelet research: i) the GPIb-V-IX complex which interacts with vWF immobilized on the injured vessel wall or on activated platelets and thereby recruits platelets from blood stream to the reactive surface under conditions of elevated shear. ii) GPIIb/IIIa (integrin aIIbβ3), a receptor for fibrinogen and vWF that requires inside-out activation mediated by agonist receptors, contributes to firm shear-resistant platelet adhesion and is essential for aggregate formation. The GPIb-V-IX complex is composed of 4 related transmembrane GPs: GPIbα, GPIbβ, GPV and GPIX, which are associated in a stoichiometry of 2:4:2:1 (Luo S-Z et al. Blood. 2007; 109(2): 603-9). Within this complex, GPIbα and GPIbβ are disulfide-linked and noncovalently associated with GPIX. GPV is noncovalently associated with GPIb-IX (Nieswandt B, et al. Journal of Thrombosis and Haemostasis. 2009; 7: 206-9). Approximately 30,000 copies of the GPIb-IX complex are found on the surface of human platelets (Varga-Szabo D, et al. Journal of Thrombosis and Haemostasis. 2009; 7: 1057-66). Loss of GPIb-V-IX function causes Bernard-Soulier syndrome (BSS), a severe bleeding disorder. BSS is characterized by abnormal, giant circulating platelets with defective adhesion to vWF and reduced thrombin responsiveness (Canobbio I, et al. Cellular signalling. 2004; 16: 1329-44). While lack or dysfunction of GPIb or GPIX are associated with BSS, no loss of function mutation in *GP5* has been reported and the lack of GPV in mice does not lead to a BSS-phenotype (Ramakrishnan V, et al. PNAS. 1999; 96: 13336-41; Kahn ML, et al. Blood. 1999; 94: 4112-21). GPV is the only subunit which is not required for the correct expression of the complex (Dong J, et al. Journal of Biological Chemistry. 1998; 273: 31449-54). WO 95/02054 A2, US 6,005,089 and Lanza F, et al. Journal of Biological Chemistry. 1993; 268: 20801-20807 disclose the sequence and structure of the human GPV gene and the amino acid sequence of human GPV. GPV is highly glycosylated and contains a thrombin cleavage site leading to quantitative removal of GPV from the platelet surface and the generation of soluble GPV (sGPV) in the presence of thrombin (Ravanat C, et al. Blood. 1997; 89: 3253-62; Azorsa DO, et al. Thrombosis and Haemostasis. 1999; 81: 131-8; White GC, et al. Thrombosis Research. 38: 641-648). The soluble human GPV generated by thrombin cleavage has the amino acid sequence as shown in SEQ ID NO:10. Of note, this thrombin cleavage site is conserved in the mouse, rat and human protein (Ravanat C, et al. Blood. 1997; 89: 3253-62). However, in contrast to protease-activated receptor (PAR) 4-deficient mice, which do not respond upon thrombin stimulation (Kahn ML, et al. Blood. 1999; 94: 4112-21; Kahn ML, et al. Nature. 1998; 394: 690-4), G*p5*$^{-/-}$ mice display grossly normal platelet functionality. Besides thrombin, GPV can, like GPIbα or GPVI, be cleaved by sheddases of the 'a disintegrin and metallopro-teinase' (ADAM) family, most notably ADAM17 (also referred to as the tumor necrosis factor-converting enzyme, TACE) and ADAM10 (Garton KJ, et al. Journal of Biological Chemistry. 2001; 276: 37993-8001; Gardiner EE, et al. Blood. 2004; 104: 3611-7; Bergmeier W, et al. Thrombosis and Haemostasis. 2004; 91: 951-8), which results in a slightly longer variant of sGPV. However, thrombin is considered as the major regulator of GPV surface expression. SGPV levels differ enormously between plasma and serum (17.3 ± 6.3 ng/ml vs. 1.2 ± 0.17 µg/ml, respectively) (Azorsa DO, et al. Thrombosis and Haemostasis. 1999; 81: 131-8) and sGPV levels are slightly elevated under certain pathological conditions, such as ischemic stroke (39.4 ng/ml compared to 28.1 ng/ml in controls) (Wolff V, et al. Stroke. 2005; 36: e17-9). To date, no role for sGPV in thrombosis or hemostasis has been described.

SUMMARY OF THE INVENTION

[0003] The inventors surprisingly found that soluble GPV has an antithrombotic effect, without affecting the bleeding time. Thus, the present invention provides an antithrombotic agent comprising soluble GPV. The present invention relates to the following embodiments (1) to (34):

1. (1) A soluble polypeptide comprising a modified glycoprotein V (GPV) lacking a functional transmembrane domain for use in the treatment and/or prevention of a thrombotic disease in a subject, said treatment and/or prevention comprising administering to the subject an effective amount of said soluble polypeptide, wherein the soluble polypeptide is incapable of being integrated into a cell membrane via said transmembrane domain, wherein the term GPV denotes a protein having a sequence identity of at least 70 % to the amino acid sequence as shown in SEQ ID No:3.
2. (2) The soluble polypeptide for use according to item (1), wherein the thrombotic disease is selected from the group consisting of thrombo-inflammatory conditions, venous thrombosis, arterial thrombosis, capillary thrombosis, portal vein thrombosis, renal vein thrombosis, jugular vein thrombosis, cerebral venous sinus thrombosis, thrombus formation during or after contacting blood with an artificial surface, in particular extracorporeal membrane oxygenation (ECMO), atherosclerosis, arthritis, coagulopathy, deep venous thrombosis (DVT), disseminated intravascular coagulopathy (DIC), a chronic or acute thromboembolism, pulmonary thrombo-embolism, Budd-Chiari syndrome, Paget-Schroetter diseases, stroke and myocardial infraction.
3. (3) The soluble polypeptide for use according to any one of the preceding items, wherein said modified GPV is a truncated GPV.
4. (4) The soluble polypeptide for use according to any one of the preceding items, wherein said modified GPV or truncated GPV consists of a fragment of the extracellular domain of a native GPV, said fragment having a length of at least 6 amino acids.
5. (5) The soluble polypeptide for use according to any one of the preceding items, wherein said modified GPV or truncated GPV consists of a fragment of the extracellular domain of a native GPV, said fragment having a length of at least 8 amino acids.
6. (6) The soluble polypeptide for use according to any one of the preceding items, wherein said modified GPV or truncated GPV consists of a fragment of the extracellular domain of a native GPV, said fragment having a length of at least 30 amino acids.
7. (7) The soluble polypeptide for use according to any one of the preceding items, wherein said modified GPV or truncated GPV consists of a fragment of the extracellular domain of a native GPV, said fragment having a length of at least 100 amino acids.
8. (8) The soluble polypeptide for use according to any one of the preceding items, wherein said modified GPV or truncated GPV consists of a fragment of the extracellular domain of a native GPV, said fragment having a length of at least 250 amino acids.
9. (9) The soluble polypeptide for use according to any one of the preceding items, wherein said modified GPV or truncated GPV consists of a fragment of the extracellular domain of a native GPV, said fragment having a length of at least 400 amino acids.
10. (10) The soluble polypeptide for use according to any one of items (4) to (9), wherein said fragment has anti-thrombotic activity.
11. (11) The soluble polypeptide for use according to any one of items (4) to (10), wherein said fragment does not substantially affect bleeding time upon administration.
12. (12) The soluble polypeptide for use according to any one of items (4) to (11), wherein

said native GPV consists of the amino acid sequence as shown in SEQ ID NO:3, and the extracellular domain substantially consists of amino acids 1-503 of SEQ ID NO:3.

13. (13) The soluble polypeptide for use according to any one of items (4) to (11), wherein said native GPV consists of the amino acid sequence as shown in SEQ ID NO:7, and the extracellular domain substantially consists of amino acids 1-502 of SEQ ID NO:7.
14. (14) The soluble polypeptide for use according to any one of the preceding items, wherein said soluble polypeptide is a non-naturally occurring polypeptide.
15. (15) The soluble polypeptide for use according to item (14), further comprising a half-life-extending moiety.
16. (16) The soluble polypeptide for use according to item (15), wherein said half-life-extending moiety is conjugated to said modified GPV, either directly or via a linker.
17. (17) The soluble polypeptide for use according to item (16), wherein said half-life-extending moiety is selected from the group consisting of hydroxyethyl starch (HES), polyethylene glycol (PEG), polysialic acids (PSAs) and albumin binding ligands, e.g. fatty acid chains.
18. (18) The soluble polypeptide for use according to item (15), wherein said half-life-extending moiety is a heterologous amino acid sequence fused to said modified GPV, either directly or via a linker.
19. (19) The soluble polypeptide for use according to item (18), wherein the half-life extending heterologous amino acid sequence comprises or consists of a polypeptide selected from the group consisting of albumin and a fragment thereof having a length of at least 100 amino acids, immunoglobulin constant regions and fragments thereof, e.g. the Fc fragment, transferrin and fragments thereof, the C-terminal peptide of human chorionic gonadotropin, solvated random chains with large hydrodynamic volume (XTEN), homo-amino acid repeats (HAP), proline-alanine-serine repeats (PAS), afamin, alpha-fetoprotein, Vitamin D binding protein, polypeptides capable of binding under physiological conditions to albumin or immunoglobulin constant regions, and combinations thereof.
20. (20) The soluble polypeptide for use according to any one of the preceding items, wherein said soluble polypeptide is obtainable by recombinant expression in eukaryotic cells.
21. (21) The soluble polypeptide for use according to item (20), wherein said eukaryotic cells are mammalian cells.
22. (22) The soluble polypeptide for use according to item (21), wherein said mammalian cells are CHO cells.
23. (23) The soluble polypeptide for use according to item (20), wherein said eukaryotic cells are insect cells, e.g. Sf9 cells.
24. (24) The soluble polypeptide for use according to any one of items (1) to (19), wherein said soluble polypeptide is obtainable by recombinant expression in prokaryotic cells, e.g. in bacterial cells.
25. (25) The soluble polypeptide for use according to any one of the preceding items, wherein said soluble polypeptide has anti-thrombotic activity.
26. (26) The soluble polypeptide for use according to any one of the preceding items, wherein said soluble polypeptide does not substantially affect bleeding time upon

administration.

27. (27) The soluble polypeptide for use according to any one of the preceding items, wherein said treatment and/or prevention further comprises administering to said subject an antiplatelet or an anticoagulant drug.
28. (28) A pharmaceutical composition comprising a soluble polypeptide as defined in any one of items (1) to (26), and a pharmaceutically acceptable excipient.
29. (29) The pharmaceutical composition of item (28), wherein the soluble polypeptide does not consist of the amino acid sequence as shown in SEQ ID NO:10.
30. (30) A method of preparing the soluble polypeptide according to any one of items (1) to (26), comprising expressing a nucleic acid encoding the soluble polypeptide as defined in any one of items (1) to (26) in a mammalian cell, and recovering the soluble polypeptide from the culture medium.
31. (31) A non-naturally occurring polypeptide as defined in item (14), wherein the modified glycoprotein V (GPV) is a non-naturally occurring soluble GPV.
32. (32) The non-naturally occurring soluble GPV of item (31), which does not consist of the amino acid sequence as shown in SEQ ID NO:10.
33. (33) A pharmaceutical kit comprising (i) a soluble polypeptide according to any one of items (1) to (26), and (ii) an antiplatelet or an anticoagulant drug other than said soluble polypeptide.
34. (34) The pharmaceutical kit of item (33), wherein the soluble polypeptide does not consist of the amino acid sequence as shown in SEQ ID NO:10.

BRIEF DESCRIPTION OF THE DRAWINGS

[0004]

Figure 1: Soluble GPV has an antithrombotic effect in an aortic injury model. The abdominal aorta was mechanically injured by a single firm compression with a forceps and blood flow was monitored with a Doppler flowmeter. Time to final occlusion is shown. A,B) Occlusion times after injection of soluble human GPV (A: shGPV; B: shGPV-Albumin-Fusion-Protein (AFP)) or soluble murine GPV (C: smGPV). Each symbol represents one individual mouse. In cases where mice were unable to form occlusive thrombi Fisher's exact test was used to calculate *P*-values. * $P<0.05$; ** $P<0.01$; *** $P<0.001$

Figure 2: Soluble human GPV protects from ischemic stroke. Mice were subjected to 60 min of transient middle cerebral artery occlusion (tMCAO). Brain infarct volumes of wildtype (black bar) and wildtype mice pretreated with shGPV-AFP (gray bar) were measured by planimetry 24 h after tMCAO. Results represent mean ± SD.

Figure 3: Soluble GPV has no effect on tail bleeding times. Displayed are tail bleeding times of the indicated mouse lines receiving either vehicle or soluble human GPV (A: shGPV; B: shGPV-AFP). Each symbol represents one animal.

Figure 4: Treatment with soluble GPV results in reduced surface coverage and thrombus volume on collagen under flow *in vitro.* A) Human blood was treated with shGPV-AFP and perfused over a collagen-coated surface at a shear rate of 1000 s^{-1}. B) Blood from wildtype mice was incubated with smGPV and perfused over a collagen-coated surface at a shear rate of 1700 s^{-1}. Results are displayed as mean ± SD.

DETAILED DESCRIPTION

[0005] The present invention relates to a soluble polypeptide comprising a modified glycoprotein V (GPV) lacking a functional transmembrane domain for use in the treatment or prevention of a thrombotic disease in a subject, said treatment or prevention comprising administering to the subject an effective amount of said soluble polypeptide, wherein the soluble polypeptide is incapable of being integrated into a cell membrane via said transmembrane domain, wherein the term GPV denotes a protein having a sequence identity of at least 70 % to the amino acid sequence as shown in SEQ ID No:3. Preferably, the modified GPV is a truncated GPV.

[0006] The term "soluble" as used herein refers to a polypeptide that is not bound to a cell membrane. The soluble polypeptide is incapable of being integrated into a cell membrane via a transmembrane domain. Typically, the soluble polypeptide lacks a functional transmembrane domain. Preferably, soluble polypeptides are soluble in water or a buffer, such as PBS.

Glycoprotein V

[0007] The term "Glycoprotein V" or "GPV", in accordance with the invention, denotes a protein having a sequence identity of at least 70% to the amino acid sequence as shown in SEQ ID NO:3. Preferably, the GPV has an amino acid identity of at least 80%, or at least 90%, or at least 95% to the amino acid sequence as shown in SEQ ID NO:3. In accordance with the present invention, a sequence being evaluated (the "Compared Sequence") has a certain "percent identity with," or is a certain "percent identical to" a claimed or described sequence (the "Reference Sequence") after alignment of the two sequences. The "Percent Identity" is determined according to the following formula:

$$\text{Percent Identity}=100[1-(C/R)]$$

[0008] In this formula, C is the number of differences between the Reference Sequence and the Compared Sequence over the length of alignment between the two sequences wherein (i) each base in the Reference Sequence that does not have a corresponding aligned base in the Compared Sequence, and (ii) each gap in the Reference Sequence, and (iii) each aligned base in the Reference Sequence that is different from an aligned base in the Compared Sequence constitutes a difference. R is the number of bases of the Reference Sequence over the length of the alignment with the Compared Sequence with any gap created in the Reference Sequence also being counted as a base.

[0009] If an alignment exists between the Compared Sequence and the Reference Sequence for which the Percent Identity (calculated as above) is about equal to, or greater than, a specified minimum, the Compared Sequence has that specified minimum Percent Identity even if alignments may exist elsewhere in the sequence that show a lower Percent Identity than that specified.

[0010] In a preferred embodiment, the length of aligned sequence for comparison purposes is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70%, 80%, or 90% of the length of the Reference Sequence.

[0011] The comparison of sequences and determination of percent identity (and percent similarity) between two amino acid sequences can be accomplished using any suitable program, e.g. the program "BLAST 2 SEQUENCES (blastp)" (Tatusova et al. FEMS Microbiol. Lett. 1999. 174: 247-250) with the following parameters: Matrix BLOSUM62; Open gap 11 and extension gap 1 penalties; gap x_dropoff50; expect 10.0 word size 3; Filter: none. According to the present invention, the sequence comparison covers at least 40 amino acids, preferably at least 80 amino acids, more preferably at least 100 amino acids, and most preferably at least 120 amino acids.

[0012] Typically, the GPV is platelet GPV, and the modified GPV is modified platelet GPV.

[0013] Native (i.e. non-modified) GPV comprises a functional transmembrane domain, i.e. it comprises an amino acid sequence capable of conferring integration into a cell membrane (e.g. a plasma membrane) during expression.

[0014] The native GPV is a naturally occurring GPV. Preferably, the native GPV is of mammalian origin. In one embodiment, the GPV is a human GPV. According to this embodiment, the native GPV preferably comprises or consists of the amino acid sequence as shown in SEQ ID NO:3. In another embodiment, the native GPV is a murine GPV. According to this embodiment, the native GPV preferably comprises or consists of the amino acid sequence as shown in SEQ ID NO:7. The term native GPV as used herein includes, but is not limited to, homologs and orthologs of human GPV represented by SEQ ID NO:2 (with signal peptide) and SEQ ID NO:3 (without signal peptide). Unless indicated otherwise, the term GPV refers to the mature polypeptide lacking the signal peptide.

[0015] Most preferably, the native GPV comprises or consists of the amino acid sequence as shown in SEQ ID NO:3.

Modified GPV

[0016] The modified GPV in accordance with this invention differs from the native GPV from which it is derived (also referred to as the "parent GPV" or "non-modified GPV") at least in that the transmembrane domain is no longer functional, due to mutation or any other means. For example, the amino acid sequence representing the transmembrane domain in the modified GPV may have one or more substitutions, deletions and/or insertions relative to the parent GPV. In one embodiment, the amino acid sequence of the modified GPV lacks at least the entire transmembrane domain of the parent GPV. In another embodiment, the modified GPV is a truncated GPV. The transmembrane domain of human GPV extends from amino acids positions 504 to 527 of SEQ ID NO:3. The transmembrane domain of murine GPV extends from amino acids positions 503 to 526 of SEQ ID NO:7.

[0017] 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23 or 24 amino acids of the transmembrane domain of the GPV may be deleted or substituted in the modified GPV. In one embodiment, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23 or 24 amino acids of the transmembrane domain of human GPV (amino acids 504 to 527 of SEQ ID NO:3) may be deleted or substituted. In another embodiment, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23 or 24 amino acids of the transmembrane domain of murine GPV (amino acids 503 to 526 of SEQ ID NO:7) may be deleted or substituted.

[0018] The modified GPV, preferably the truncated GPV, has antithrombotic activity. Antithrombotic activity can be determined as shown in the experiment described in Example 1 (See also "Mechanical Injury of the Abdominal Aorta" in the "Materials and Methods" section of the Examples). There is antithrombotic activity if the tested compound (e.g. 20 µg) is capable of delaying or preventing arterial occlusive thrombus formation in mice. Preferably, the arterial occlusive thrombus formation is delayed by at least 1 minute, more preferably by at least 5 minutes, most preferably by at least 10 minutes.

Truncated GPV

[0019] A truncated GPV consists of a fragment of GPV. The truncation typically is at the C-terminal end of the GPV. The N-terminal end may be truncated, or it may not be truncated.

[0020] The fragment of GPV has a length of at least 6 amino acids. Preferably, the length of the GPV fragment is at least 7, at least 8, at least 9, at least 10, at least 15, at least 20, at least 50, at least 100, at least 200, at least 300, or at least 400 amino acids. In certain embodiments, the truncated GPV consists of a fragment of the amino acid sequence as shown in SEQ ID NO:3, wherein said fragment has a minimum length of 6 amino acids, preferably of at least 7, at least 8, at least 9, at least 10, at least 15, at least 20, at least 50, at least 100, at least 200, at least 300, or at least 400 amino acids.

[0021] In one embodiment, the truncated GPV has a C-terminal truncation and lacks the complete transmembrane domain of the GPV from which it is derived. In another embodiment, the truncated GPV has a C-terminal truncation and lacks the transmembrane domain to such an extent that the truncated GPV is not membrane-bound.

[0022] Preferred truncated GPVs consist of an amino acid sequence selected from the following amino acid sequences, wherein all amino acid positions refer to SEQ ID NO:3 (embodiments 1-71) or SEQ ID NO:7 (embodiments 72-141), respectively, as indicated:

Table 1.

Embodiment No.	from	to		Embodiment No.	from	to
	of SEQ ID NO:3				of SEQ ID NO:7	
1	1	520		72	1	520
2	2	519		73	2	519
3	3	518		74	3	518
4	4	517		75	4	517
5	5	516		76	5	516
6	6	515		77	6	515
7	7	514		78	7	514
8	8	513		79	8	513
9	9	512		80	9	512
10	10	511		81	10	511
11	11	510		82	11	510
12	12	509		83	12	509
13	13	508		84	13	508
14	14	507		85	14	507
15	15	506		86	15	506
16	16	505		87	16	505
17	17	504		88	17	504
18	18	503		89	18	503
19	19	502		90	19	502
20	20	501		91	20	501
21	21	500		92	21	500
22	22	499		93	22	499
23	23	498		94	23	498
24	24	497		95	24	497
25	25	496		96	25	496

Embodiment No.	from	to		Embodiment No.	from	to
	of SEQ ID NO:3				of SEQ ID NO:7	
26	26	495		97	26	495
27	27	494		98	27	494
28	28	493		99	28	493
29	29	492		100	29	492
30	30	491		101	30	491
31	31	490		102	31	490
32	32	489		103	32	489
33	33	488		104	33	488
34	34	487		105	34	487
35	35	486		106	35	486
36	36	485		107	36	485
37	37	484		108	37	484
38	38	483		109	38	483
39	39	482		110	39	482
40	40	481		111	40	481
41	41	480		112	41	480
42	42	479		113	42	479
43	43	478		114	43	478
44	44	477		115	44	477
45	45	476		116	45	476
46	46	475		117	46	475
47	47	474		118	47	474
48	48	473		119	48	473
49	49	472		120	49	472
50	50	471		121	50	471
51	51	470		122	51	470
52	52	469		123	52	469
53	53	468		124	53	468
54	54	467		125	54	467
55	55	466		126	55	466
56	56	465		127	56	465
57	57	464		128	57	464
58	58	463		129	58	463

Embodiment No.	from	to		Embodiment No.	from	to
	of SEQ ID NO:3				of SEQ ID NO:7	
59	59	462		130	59	462
60	60	461		131	60	461
61	61	460		132	61	460
62	62	459		133	62	459
63	63	458		134	63	458
64	64	457		135	64	457
65	65	456		136	65	456
66	66	455		137	66	455
67	67	454		138	67	454
68	68	453		139	68	453
69	69	452		140	69	452
70	70	451		141	70	451
71	71	450		142	71	450

[0023] The upper and lower limits of the amino acid sequences of above embodiments can be combined with each other.

[0024] In particularly, preferred embodiments of the truncated GPV consists of amino acids 1-516 of SEQ ID NO:3, or of amino acids 1-502 of SEQ ID NO:7.

[0025] In other embodiments, the truncated GPV comprises or consist of the following sequences.

Table 2.

Embodiment No.	The truncated GPV comprises or consists of the following amino acids of SEQ ID NO:3
143	1-15
144	16-30
145	31-45
146	46-60
147	61-75
148	76-90
149	91-105
150	106-120
151	121-135
152	136-150

Embodiment No.	The truncated GPV comprises or consists of the following amino acids of SEQ ID NO:3
153	151-165
154	166-180
155	181-195
156	196-205
157	211-225
158	226-240
159	241-255
160	256-270
161	271-285
162	286-300
163	301-315
164	316-330
165	331-345
166	346-360
167	361-365
168	376-390
169	391-405
170	406-420
171	421-435
172	436-450
173	451-465
174	466-480
175	481-500

[0026] In a specific embodiment of the present invention the soluble polypeptide for use as described herein comprises or consists of the amino acid sequence as shown in SEQ ID NO:10.

[0027] In another specific embodiment the soluble polypeptide of the invention is a non-naturally occurring polypeptide. In yet another specific embodiment the soluble polypeptide of the invention does not consist of the amino acid sequence as shown in SEQ ID NO:10. In yet another specific embodiment the soluble polypeptide of the invention is a non-naturally occurring polypeptide and does not consist of the amino acid sequence as shown in SEQ ID NO:10.

Further components of the polypeptide

[0028] The soluble polypeptide of the invention may comprise additional amino acids other than those derived from GPV or other half-life extending moieties.

[0029] In one embodiment of the invention, the half-life of the soluble polypeptide of the invention is extended by chemical modification, e.g. attachment, either directly or via a linker, 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 soluble polypeptide, preferably the modified GPV, is conjugated to a half-life extending protein (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).

[0030] In another embodiment of the invention, the soluble polypeptide comprises a heterologous amino acid sequence, i.e. heterologous to the respective GPV used, which is fused to said GPV either directly or via a linker.

[0031] Heterologous sequences may be tag sequences which are recognized by antibodies or other molecules having high affinity to the tag. Examples include, but are not limited to, polyhistidine tags, FLAG tag, myc-tag, GST tag, etc. Tag sequences usually facilitate purification of the polypeptide upon expression in host cells.

[0032] In a preferred embodiment, the soluble polypeptide further comprises a half-life extending protein (HLEP). Preferably, the HLEP is an albumin or a fragment thereof. The N-terminus of the albumin may be fused to the C-terminus of the modified GPV. One or more HLEPs may be fused to the N- or C-terminal part of modified GPV provided that they do not interfere with or abolish the anti-thrombotic activity of the modified GPV.

[0033] In one embodiment the polypeptide has the following structure:

mGPV - L1 - H, [formula 1]

wherein mGPV is the modified GPV, L1 is a chemical bond or a linker sequence, and H is a HLEP.

[0034] 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 GPV. 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 modified GPV 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.

[0035] Preferred HLEP sequences are described below. Likewise encompassed by the invention are fusions to the exact "N-terminal amino acid" of the respective HLEP, or fusions to the "N-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 modified GPV in tandem, e.g. as successive repeats.

Half-life extending polypeptides (HLEPs)

[0036] 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 fragments thereof, e.g. the Fc fragment, solvated random chains with large hydrodynamic volume (e.g. XTEN (Schellenberger et al. Nature Biotechnol. 2009. 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.

[0037] A "half-life extending 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 fragments of an immunoglobulin constant region. It may be a full-length half-life-extending 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 modified GPV. 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.

[0038] The HLEP fragment of the proposed coagulation factor insertion constructs of the invention may be a variant of a normal HLEP. The term "variants" includes insertions, deletions and substitutions, either conservative or non-conservative, where such changes do not substantially alter the active site, or active domain which confers the biological activities of the modified GPV.

[0039] In particular, the proposed modified GPV-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.

Albumin as HLEP

[0040] 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).

[0041] 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 or albumin from other vertebrates or fragments thereof, or analogs or variants of these molecules or fragments thereof.

[0042] In particular, the proposed modified GPV fusion constructs of the invention may include 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.

[0043] The albumin fragment of the proposed modified GPV fusion constructs of the invention may comprise at least one subdomain or domain of HA or conservative modifications thereof.

Immunoglobulins as HLEPs

[0044] In a preferred embodiment the soluble polypeptide of the invention comprises or consists of the amino acid sequence as shown in SEQ ID NO:9.

Immunoglobulins as HLEPs

[0045] Immunoglobulin G (IgG) constant regions (Fc) are known in the art to increase the half-life of therapeutic proteins (Dumont JA, et al. BioDrugs. 2006; 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 fragment 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 proteins in vivo half-lives. US 2004/0087778 and WO 2005/001025 describe fusion proteins of Fc domains or at least fragments 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 (US 6,403,077), all with half-life extending properties.

[0046] In certain embodiments, the Fc fusion protein is monomeric. In other embodiments, the Fc fusion protein is dimeric.

[0047] Various HLEPs which can be used in accordance with this invention are described in detail in WO 2013/120939.

Nucleic Acid and Expression

[0048] The nucleic acid encoding the polypeptide to be expressed can be prepared according to methods known in the art. Based on the cDNA sequence of human GPV (SEQ ID NO:1) or of murine GPV (SEQ ID NO:5), recombinant DNA encoding the above-mentioned modified GPV constructs can be designed and generated. Further details of the human and mouse sequences are summarized in table 3.

Table 3: Details of mouse and human wild-type GPV Sequences

	Mouse	Human
Entrez	14729	2814
Ensembl	ENSMUSG00000047953	ENSG00000178732
Uniprot	Q3TA66	P40197
mRNA RefSeq	NM_008148	NM_004488
Protein RefSeq	NP_032174	NP_004479
Location	Chr 16: 30.23 - 30.23 Mb	Chr 3: 195.6 - 195.6 Mb
Molecular Mass [Da]	63251	60828

[0049] Constructs in which the cDNA 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.

[0050] Typically, the cells to be provided are obtained by introducing the nucleic acid encoding a polypeptide comprising the modified GPV into mammalian host cells.

Host cells

[0051] Any host cell susceptible to cell culture, and to expression of polypeptides, preferably glycosylated polypeptides, may be utilized in accordance with the present invention. In certain embodiments, the 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 (HEK293 or HEK293 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 human or a rodent cell line, especially a hamster cell line such as CHO.

[0052] 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. 1981; 293: 620-625; Mantei, et al., Nature. 1979; 281: 40-46; 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. 1978; 52: 456-457) or the lipofectamine™ (Gibco BRL) Method of Hawley-Nelson (Focus. 1993; 15: 73). General aspects of mammalian host cell system transfections have been described by Axel in US 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. 1990; 185: 527-537), and Mansour et al. (Nature, 1988; 336: 348-352).

[0053] 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 production medium is free of animal-derived components. In a preferred embodiment, the medium is "protein-free" in the sense that it is either completely free of any protein or at least 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 glycoprotein. Optionally, 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.

[0054] Generally, the present invention may be used with any cell culture method that is amenable to the expression of glycoproteins. For example, cells may be grown in batch or fed-batch cultures, where the culture is terminated after sufficient expression of the glycoprotein, after which the expressed glycoprotein is harvested. Preferably, cells may be grown in continuous cultures (e.g. perfusion cultures), where fresh medium is periodically or continuously added to the culture, and the expressed glycoprotein is harvested periodically or continuously. The culture can be of any conventional type of culture, such as batch, fed-batch or continuous, but is preferably continuous. Suitable continuous cultures include perfusion culture.

[0055] 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 glycoprotein, 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 soluble polypeptide

[0056] In general, it will typically be desirable to isolate and/or purify glycoproteins expressed according to the present invention. In certain embodiments, the expressed glycoprotein 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.

[0057] The expressed glycoprotein 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 SJ and Hames BD (eds.), Protein Expression: A Practical Approach, Oxford Univ Press, 1999; and Deutscher MP, Simon MI, Abelson JN (eds.), Guide to Protein Purification:

Methods in Enzymology (Methods in Enzymology Series, Vol. 182), Academic Press, 1997, each of which is incorporated herein by reference). For immunoaffinity chromatography in particular, the glycoprotein may be isolated by binding it to an affinity column comprising antibodies that were raised against that glycoprotein and were affixed to a stationary support. Alternatively, affinity tags such as an influenza coat sequence, polyhistidine, or glutathione-S-transferase can be attached to the glycoprotein by standard recombinant techniques to allow for easy purification by passage over the appropriate affinity column. If the soluble GPV to be purified comprises a HLEP, antibodies directed against the HLEP, or other compounds capable of binding to the HLEP can be affixed to an affinity column so as to perform affinity chromatography. 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 glycoprotein during the purification process. Protease inhibitors are particularly advantageous when cells must be lysed in order to isolate and purify the expressed glycoprotein. 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.

[0058] 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.

Compositions and Kits

[0059] Another aspect of the invention is a pharmaceutical composition comprising soluble polypeptide of the invention, and a pharmaceutically acceptable excipient or carrier. The pharmaceutical composition may comprise a soluble polypeptide in an effective amount for treating or preventing a thrombotic disease in a subject. The pharmaceutical composition may comprise about 10 µg - 1,000 mg, preferably about 100 µg - 500 mg, more preferably 1 mg - 100 mg of the soluble polypeptide. The pharmaceutical composition may comprise about 0.01 - 20,000 µg/ml, preferably about 0.1 - 1000 µg/ml, more preferably 0.5 - 500 µg/ml, most preferably about 100 µg/ml of the soluble polypeptide. The pharmaceutical composition may further comprise a pharmaceutically acceptable carrier or diluent. Carriers and diluents suitable in the pharmaceutical composition are well known in the art.

[0060] Therapeutic formulations of the glycoproteins of the invention suitable in the methods described herein can be prepared for storage as lyophilized formulations or aqueous solutions by mixing the glycoprotein 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, isotonifiers, 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. Buffering agents help to maintain the pH in the range which approximates physiological conditions. They can 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 glyuconate 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.

[0061] 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, myoinisitol, galactitol, glycerol and the like, including cyclitols such as inositol; polyethylene glycol; amino acid polymers; sulfur containing reducing agents, such as urea, glutathione, thioctic 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; hydrophylic polymers, such as polyvinylpyrrolidone monosaccharides, such as xylose, mannose, fructose, glucose; disaccharides such as lactose, maltose, sucrose and trisaccacharides 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.

[0062] 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.

[0063] Additional miscellaneous excipients include bulking agents (e.g. starch), chelating agents (e.g. EDTA), antioxidants (e.g. ascorbic acid, methionine, vitamin E), and cosolvents. The pharmaceutical composition may have a pH of about 5.0-10.0, preferably about 5.6-9.0, more preferably about 6.0-8.8, most preferably about 6.5-8.0. For example, the pH may be about 6.2, 6.5, 6.75, 7.0, or 7.5.

[0064] The pharmaceutical compositions of the present invention may be formulated for oral, sublingual, intranasal, intraocular, rectal, transdermal, mucosal, topical or parenteral administration. Parenteral administration may include intradermal, subcutaneous, intramuscular (i.m.), intravenous (i.v.), intraperitoneal (i.p.), intra-arterial, intramedullary, intracardiac, intraarticular (joint), intrasynovial, intracranial, intraspinal, and intrathecal (spinal fluids) injection or infusion, preferably intraperitoneal (i.p.) injection in mouse and intravenous (i.v.) or subcutaneous (s.c.) in human. Any device suitable for parenteral injection or infusion of drug formulations may be used for such administration. For example, the pharmaceutical composition may be contained in a sterile pre-filled syringe.

[0065] Another aspect of the present invention is a pharmaceutical kit comprising (i) a soluble polypeptide as defined hereinabove and (ii) an anticoagulant or antiplatelet drug other than said soluble polypeptide. In one embodiment, the soluble polypeptide and the anticoagulant or antiplatelet drug are contained in separate compositions.

[0066] The term "anticoagulant or antiplatelet drug" refers to heparins, direct thrombin inhibitors (DTI), direct or selective Factor Xa inhibitors (xaban) and vitamin K antagonists (VKA). Thus, "anticoagulant or antiplatelet drugs" can include natural occurring or synthetic heparins. The term "anticoagulant or antiplatelet drug" also meant to include substances that prevent coagulation of blood by inhibiting directly or selective thrombin or Factor Xa. In another embodiment, the anticoagulant substance is a vitamin K antagonist.

[0067] In some embodiments the anticoagulant or antiplatelet drug is selected from

1. (i) a heparin, in particular a unfractionated heparin (UFH) or a low-molecular-weight heparin (LMWH),
2. (ii) a direct thrombin inhibitor (DTI), in particular dabigatran, melagatran, argatroban, hirudin, lepirudin, bivalirudin, ximelagatran or desirudin (Di Nisio et al. N Engl J Med. 2005; 353: 1028-40),
3. (iii) a direct or selective Factor Xa inhibitor (xaban), in particular rivaroxaban (Eriksson et al., Circulation. 114: 2374-81), apixaban (Arterioscler. Thromb. Vasc. Biol. 27: 1238-47),

betrixaban, edoxaban, otamixaban (Cohen et al., Circulation 115: 2642-51) or fondaparinux (Peters et al., Eur. Heart J. 29: 324-31) and,

4. (iv) a vitamin K antagonist (VKA), in particular phenprocoumon, acenocoumarol or warfarin and related 4-hydroxycoumarin-containing molecules, coumatetralyl, dicoumarol, ethyl biscoumacetate, clorindione, diphenandione, phenandione or tioclomarol (see e.g. Ansell et al. 2008, "Pharmacology and management of the vitamin K antagonists", American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition). Chest 133 (6 Suppl): 160S-198S).

[0068] Another aspect of the present invention is a pharmaceutical kit comprising (i) a soluble polypeptide as defined hereinabove and (ii) an antiplatelet or anticoagulant drug other than said soluble polypeptide, for simultaneous, separate or sequential use in the treatment of a thrombotic disease.

Treatment of thrombotic disease

[0069] The soluble polypeptide of the invention can be used for treating or preventing thrombotic diseases.

[0070] A "thrombotic disorder" or "thrombotic disease" used herein is any disorder or disease characterized by the formation of a thrombus (blood clot) that obstructs or decreases blood flow. The thrombus may remain local to where it formed, or it may detach to occlude blood flow downstream (thromboembolism). In some embodiments, a thrombosis may occur in a vein (venous thrombosis) or in an artery (arterial thrombosis) anywhere in the body, including the heart and brain. When the thrombosis occurs in the coronary circulation, it is referred to as a coronary thrombosis. When the thrombosis occurs in the cerebral circulation, it is referred to as a cerebral thrombosis.

[0071] A thrombotic disorder can include a venous, arterial, or capillary thrombosis, thrombus formation in the heart, chronic and/or acute thromboembolism (e.g. pulmonary embolism, cerebral thromboembolism following atrial fibrillation-induced thrombus formation (e.g. stroke prevention in atrial fibrillation (SPAF)), thrombus formation as a result of contacting the blood of a human or animal subject with an artificial surface (e.g. in patients with valve replacements, in particular a mechanical heart valve, stents, percutaneous coronary intervention (PCI), extracorporeal membrane oxygenation (ECMO), or undergoing cardiopulmonary bypass surgery (CPB surgery)). The thrombus can cause or increase the risk of a stroke, acute ischemic stroke, myocardial infarction, unstable angina, deep vein thrombosis (DVT), portal vein thrombosis, thromboembolism, renal vein thrombosis, jugular vein thrombosis, cerebral venous sinus thrombosis, Budd-Chiari syndrome, Paget-Schroetter diseases, or silent brain ischemia (SBI). A thrombotic disease in accordance with this invention may further include pulmonary embolism, atherosclerosis, factor V Leiden, antithrombin III deficiency, protein C deficiency, protein S deficiency, prothrombin gene mutation (G20210A), hyperhomocysteinemia, antiphospholipid antibody syndrome, anticardiolipin antibody, thrombosis syndrome, lupus anticoagulant syndrome, malignancy, major surgery, immobilization, oral contraceptive use, thalidomide use, especially in combination with dexamethasone, heparin-induced thrombocytopenia, pregnancy, myeloproliferative disorders, inflammatory bowel disease, nephrotic syndrome, paroxysmal nocturnal hemoglobinuria, hyperviscosity syndrome, Waldenstrom's macroglobulinemia, and trauma. The term thrombotic disease also refers to thrombosis induced by cancer, e.g. multiple myeloma and other hematologic cancers, adenocarcinoma, cancer of the pancreas, stomach, ovaries, prostate, colon, lung, brain, breast, kidney, skin, cervix, and ear-nose-throat cancer.

[0072] The term "thrombotic disease" particularly includes thrombo-inflammatory conditions. Thrombo-inflammation means disease states, where prothrombotic and pro-inflammatory cascades act in concert and are mechanistically linked to promote disease progression and organ damage. Thrombo-inflammatory disease states include conditions of post-ischemic organ damage, such as ischemia/reperfusion injury (I/R-injury) of the brain (in acute ischemic stroke), lung, liver, colon, myocardium, or skeletal muscle but also systemic inflammatory conditions such as sepsis or septic shock.

[0073] Preferably, the thrombotic disease is selected from the group consisting of thrombo-inflammatory conditions, venous thrombosis, arterial thrombosis, capillary thrombosis, portal vein thrombosis, renal vein thrombosis, jugular vein thrombosis, cerebral venous sinus thrombosis, thrombus formation during or after contacting blood with an artificial surface, in particular extracorporeal membrane oxygenation (ECMO), atherosclerosis, arthritis, coagulopathy, deep venous thrombosis (DVT), disseminated intravascular coagulopathy (DIC), a chronic or acute thromboembolism, pulmonary thromboembolism, Budd-Chiari syndrome, Paget-Schroetter diseases, stroke and myocardial infraction.

[0074] Determination of the effective dosage, total number of doses, and length of treatment with a soluble polypeptide of the invention is well within the capabilities of those skilled in the art, and can be determined using a standard dose escalation study. The dosage of a soluble polypeptide of the invention to be administered will vary according to the particular soluble 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.

[0075] The dosing schedule can vary from once a month to daily depending on a number of clinical factors, including the particular type of disease, severity of disease, and the patient's sensitivity to the soluble polypeptide of the invention. In specific embodiments, a soluble 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 week to every month, from every 10 days to every two weeks, or from two to three times a week, etc.

[0076] It will be recognized by one of skill in the art that the optimal quantity and spacing of individual dosages of a soluble 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.

Table 4: Overview of the sequences shown in the sequence listing

SEQ ID NO:	Description
1	cDNA encoding human GPV
2	Amino acid sequence encoded by SEQ ID NO:1; amino acids 1-16 represent the signal peptide
3	Amino acid sequence of human GPV without signal peptide
4	Amino acid sequence of soluble human GPV with poly-His tag (without signal peptide)
5	cDNA encoding murine GPV
6	Amino acid sequence encoded by SEQ ID NO:5; amino acids 1-16 represent the signal peptide
7	Amino acid sequence of murine GPV without signal peptide
8	Amino acid sequence of soluble murine GPV with Flag-tag (without signal peptide)
9	Amino acid sequence of soluble human GPV fused to albumin via linker (without signal peptide)
10	Amino acid sequence of naturally occurring thrombin cleavage product of human GPV

EXAMPLES

__Results__

Example 1: Soluble GPV has an Antithrombotic Effect

[0077] In order to investigate a potential effect of sGPV on *in vivo* thrombus formation in a model of mechanical injury of the abdominal aorta, 20 µg human sGPV (shGPV) were injected intravenously into WT mice directly before the experiment. Within 8 min after the aortic injury, blood flow stopped due to occlusive thrombus formation in PBS-injected control mice (Fig 1).

Pretreatment with sGPV protected WT mice from arterial occlusive thrombus formation indicating that the shGPV has an antithrombotic effect (Figure 1).

Example 2: Soluble human GPV protects from ischemic stroke

[0078] To assess the role of soluble GPV in brain infarction after focal cerebral ischemia, mice were subjected to 60-minute transient middle cerebral artery occlusion (tMCAO), and infarct volume was assessed after 24 hours. Strikingly, the infarct volumes in wildtype mice treated with shGPV-AFP were significantly reduced compared with wild-type mice (Figure 2). Thus, pre-treatment with soluble human GPV provides protection against cerebral infarct progression.

Example 3: Soluble human GPV has no effect on tail bleeding times.

[0079] To assess the role of soluble GPV on hemostasis, mice treated either with vehicle or 20 µg soluble human GPV (shGPV) were subjected to the tail bleeding time assay. A 2-mm segment of the tail tip was removed with a scalpel. Tail bleeding was monitored by gently absorbing blood with filter paper at 20 s intervals without directly contacting the wound site. When no blood was observed on the paper, bleeding was determined to have ceased. (Figure 3). These data demonstrate, that shGPV doses, which exert anti-thrombotic effects (Figure 1) do not affect hemostasis indicating that shGPV is a safe anti-thrombotic agent.

Example 4: Adhesion to collagen under flow *in vitro*

[0080] To assess the role of soluble GPV on thrombus formation on collagen under flow in an in vitro assay, anticoagulated whole blood was incubated with 20 µg soluble GPV for 5 min and perfused over a collagen-coated surface. Human blood pretreated with shGPV-AFP (A) or wildtype blood pretreated with soluble murine GPV (B) exhibited a significantly reduced surface coverage and reduced thrombus formation. Thus, *in vitro* flow adhesion assay models the *in vivo* conditions too a large extent and reproduces the *in vivo* phenotype.

Materials and Methods for Examples 1-4

Mice

[0081] Animal studies were approved by the district government of Lower Franconia (Bezirksregierung Unterfranken).

Soluble GPV

1. Soluble human GPV (shGPV)

[0082] Soluble human GPV (aa 1-518 of mature human GPV) was recombinantly expressed in baculovirus-transfected insect cells, purified using a standard nitrilotriacetic acid (Ni-NTA) column and solved in PBS buffer. Purity was checked using standard SDS PAGE.

[0083] Amino acid sequence of mature shGPV (signal peptide not shown):
QPFPCPPACKCVFRDAAQCSGGDVARISALGLPTNLTHILLFGMGRGVLQSQSFSGMTVLQRLMISDSHISAVAP
GTFSDLIKLKTLRLSRNKITHLPGALLDKMVLLEQLFLDHNALRGIDQNMFQKLVNLQELALNQNQLDFLPASLF
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HH (SEQ ID NO:4)
(Italics: poly-His tag)

2. Soluble human GPV fused to albumin (shGPV-AFP)

[0084] The shGPV-AFP was expressed in CHO K1 cells and produced in a perfusion fermenter system. The cell free harvest was 30 fold concentrated using a TFF system (e.g. Centramate 500 S Pall) with a 30 kD membrane (e.g Centramate OS030T12). That concentrate was spiked with NaCl and EDTA to a final concentration of 0.75 mol/L NaCl and 5 mmol/L EDTA and loaded overnight on a CaptureSelect Human Albumin column (Lifetechnologies) which was preequlibrated with 20 mM Tris buffer pH 7.4. After washing the column with equilibration buffer shGPV-AFP was eluted with 20 mM Tris plus 2 M MgCl pH 7.4 buffer. The eluate was than concentrated and dialyzed against 50 mM Tris + 150 mM NaCl pH7.4 using Ultra Centrifugal Filters with a 30 kD cut off (e.g. Amicon Ref. UFC903024).

[0085] Amino acid sequence of mature shGPV-AFP (signal peptide not shown):
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NRRPCFSALEVDETYVPKEFNAETFTFHADICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEK
CCKADDKETCFAEEGKKLVAASQAALGL (SEQ ID NO:9)
(Underlined: thrombin cleavage site and GGS Linker; Italics: human albumin sequence)

3. Soluble murine GPV (smGPV)

[0086] Soluble murine GPV (aa 1-519 of mature murine GPV) was recombinantly expressed in CHO cells, purified using an anti-Flag column and solved in PBS buffer. Purity was checked using standard SDS PAGE.

[0087] Amino acid sequence of mature smGPV (signal peptide not shown):
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DK (SEQ ID NO:8)
(Italics: Flag tag)

Mechanical-Injury of the Abdominal Aorta

[0088] To open the abdominal cavity of anesthetized mice (10-16 weeks of age), a longitudinal midline incision was performed and the abdominal aorta was exposed. A Doppler ultrasonic flow probe (Transonic Systems, Maastricht, Netherlands) was placed around the aorta and thrombosis was induced by a mechanical injury with a single firm compression (15 s) of a forceps upstream of the flow probe. Blood flow was monitored until complete occlusion occurred or 30 min had elapsed.

Transient Middle Cerebral Artery Occlusion (tMCAO)

[0089] Focal cerebral ischemia was induced in 8-to-12-week-old mice by a transient middle cerebral artery occlusion (tMCAO). Inhalation anesthesia was induced by 2% isoflurane in a 70% N_2/30% O_2 mixture and a servo-controlled heating device was used to record and maintain body temperature during the surgical procedure. The duration of the surgical procedure per animals was kept below 15 minutes. A silicon rubber-coated 6.0 nylon monofilament (6021PK10, Doccol, Redlands, CA, USA) was advanced through the carotid artery up to the origin of the middle cerebral artery (MCA) causing an MCA infarction. After an occlusion time of 60 min, the filament was removed allowing reperfusion. Animals were sacrificed 24 h after reperfusion and brains were checked for intracerebral hemorrhages. The extent of infarction was quantitatively assessed 24 hours after reperfusion on 2,3,5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich) (2% (w/v) solution) stained brain sections. Planimetric measurements of infarcted areas (ImageJ software, NIH, Bethesda, MD, USA) corrected for brain edema were performed in a blinded fashion.

Bleeding Time Assay

[0090] Mice were anesthetized by intraperitoneal injection of triple anesthesia and a 2-mm segment of the tail tip was removed with a scalpel. Tail bleeding was monitored by gently absorbing blood with filter paper at 20 s intervals without directly contacting the wound site. When no blood was observed on the paper, bleeding was determined to have ceased. The experiment was manually stopped after 20 min by cauterization.

Thrombus Formation on Collagen Under Flow *In Vitro*

[0091] For adhesion to collagen, coverslips were coated with 200 µg mL^{-1} collagen I at 37°C o/n and blocked for 1 h with 1% BSA in PBS. Whole blood (700 µl + 300 µl heparin (20 U/ml in TBS, pH7.3)) was diluted 2:1 in Tyrode's buffer containing Ca^{2+} and filled into a 1 ml syringe. Before perfusion, anticoagulated blood was incubated with Dylight-488-conjugated anti-GPIX derivative (0.2 µg/mL) at 37°C for 5 minutes. Transparent flow chambers with a slit depth of 50 µm, equipped with the coated coverslips, were connected to a syringe that was filled with diluted whole blood. Perfusion was performed using a pulse-free pump under high shear stress equivalent to a wall shear rate of 1000 s^{-1} or 1,700 s^{-1}. Aggregate formation was visualized with a Zeiss Axiovert 200 inverted microscope (40x/0.60 objective). Phase-contrast and fluorescence pictures were recorded with a CoolSNAP-EZ camera, and analyzed off-line using MetaVue software.

Statistical Analysis

[0092] Results are shown as mean ± SD from at least three individual experiments per group. When applicable Fisher's exact test was used for statistical analysis. Otherwise, the Welch's t test was performed for statistical analysis. P-values <0.05 were considered statistically significant.

SEQUENCE LISTING

[0093]

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<120> Soluble Glycoprotein V as antithrombotic agent

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<150> EP15202530.0
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Ala Cys Lys Cys Val Phe Arg Asp Ala Ala Gln Cys Ser Gly Gly Asp
    25                  30                  35

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200                 205                 210                 215

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<400> 2
Met Leu Arg Gly Thr Leu Leu Cys Ala Val Leu Gly Leu Leu Arg Ala
1 5 10 15

Gln Pro Phe Pro Cys Pro Pro Ala Cys Lys Cys Val Phe Arg Asp Ala
20 25 30

Ala Gln Cys Ser Gly Gly Asp Val Ala Arg Ile Ser Ala Leu Gly Leu
35 40 45

Pro Thr Asn Leu Thr His Ile Leu Leu Phe Gly Met Gly Arg Gly Val
50 55 60

Leu Gln Ser Gln Ser Phe Ser Gly Met Thr Val Leu Gln Arg Leu Met
65 70 75 80

Ile Ser Asp Ser His Ile Ser Ala Val Ala Pro Gly Thr Phe Ser Asp
85 90 95

Leu Ile Lys Leu Lys Thr Leu Arg Leu Ser Arg Asn Lys Ile Thr His
100 105 110

Leu Pro Gly Ala Leu Leu Asp Lys Met Val Leu Leu Glu Gln Leu Phe
115 120 125

Leu Asp His Asn Ala Leu Arg Gly Ile Asp Gln Asn Met Phe Gln Lys
130 135 140

Leu Val Asn Leu Gln Glu Leu Ala Leu Asn Gln Asn Gln Leu Asp Phe
145 150 155 160

Leu Pro Ala Ser Leu Phe Thr Asn Leu Glu Asn Leu Lys Leu Leu Asp
165 170 175

Leu Ser Gly Asn Asn Leu Thr His Leu Pro Lys Gly Leu Leu Gly Ala

Leu Ser Gly Asn Asn Leu Thr His Leu Pro Lys Gly Leu Leu Gly Ala
180 185 190

Gln Ala Lys Leu Glu Arg Leu Leu Leu His Ser Asn Arg Leu Val Ser
195 200 205

Leu Asp Ser Gly Leu Leu Asn Ser Leu Gly Ala Leu Thr Glu Leu Gln
210 215 220

Phe His Arg Asn His Ile Arg Ser Ile Ala Pro Gly Ala Phe Asp Arg
225 230 235 240

Leu Pro Asn Leu Ser Ser Leu Thr Leu Ser Arg Asn His Leu Ala Phe
245 250 255

Leu Pro Ser Ala Leu Phe Leu His Ser His Asn Leu Thr Leu Leu Thr
260 265 270

Leu Phe Glu Asn Pro Leu Ala Glu Leu Pro Gly Val Leu Phe Gly Glu
275 280 285

Met Gly Gly Leu Gln Glu Leu Trp Leu Asn Arg Thr Gln Leu Arg Thr
290 295 300

Leu Pro Ala Ala Ala Phe Arg Asn Leu Ser Arg Leu Arg Tyr Leu Gly

305 310 315 320

Val Thr Leu Ser Pro Arg Leu Ser Ala Leu Pro Gln Gly Ala Phe Gln
325 330 335

Gly Leu Gly Glu Leu Gln Val Leu Ala Leu His Ser Asn Gly Leu Thr
340 345 350

Ala Leu Pro Asp Gly Leu Leu Arg Gly Leu Gly Lys Leu Arg Gln Val
355 360 365

Ser Leu Arg Arg Asn Arg Leu Arg Ala Leu Pro Arg Ala Leu Phe Arg
370 375 380

Asn Leu Ser Ser Leu Glu Ser Val Gln Leu Asp His Asn Gln Leu Glu
385 390 395 400

Thr Leu Pro Gly Asp Val Phe Gly Ala Leu Pro Arg Leu Thr Glu Val
405 410 415

Leu Leu Gly His Asn Ser Trp Arg Cys Asp Cys Gly Leu Gly Pro Phe
420 425 430

Leu Gly Trp Leu Arg Gln His Leu Gly Leu Val Gly Gly Glu Glu Pro
435 440 445

Pro Arg Cys Ala Gly Pro Gly Ala His Ala Gly Leu Pro Leu Trp Ala
450 455 460

Leu Pro Gly Gly Asp Ala Glu Cys Pro Gly Pro Arg Gly Pro Pro Pro
465 470 475 480

Arg Pro Ala Ala Asp Ser Ser Ser Glu Ala Pro Val His Pro Ala Leu
                485   490   495

Ala Pro Asn Ser Ser Glu Pro Trp Val Trp Ala Gln Pro Val Thr Thr
            500   505   510

Gly Lys Gly Gln Asp His Ser Pro Phe Trp Gly Phe Tyr Phe Leu Leu
        515   520   525

Leu Ala Val Gln Ala Met Ile Thr Val Ile Ile Val Phe Ala Met Ile
    530   535   540

Lys Ile Gly Gln Leu Phe Arg Lys Leu Ile Arg Glu Arg Ala Leu Gly
545   550   555   560

<210> 3

<211> 544

<212> PRT

<213> Homo sapiens

<400> 3

Gln Pro Phe Pro Cys Pro Pro Ala Cys Lys Cys Val Phe Arg Asp Ala
1   5   10   15

Ala Gln Cys Ser Gly Gly Asp Val Ala Arg Ile Ser Ala Leu Gly Leu
            20   25   30

Pro Thr Asn Leu Thr His Ile Leu Leu Phe Gly Met Gly Arg Gly Val
        35   40   45

Leu Gln Ser Gln Ser Phe Ser Gly Met Thr Val Leu Gln Arg Leu Met
    50   55   60

Ile Ser Asp Ser His Ile Ser Ala Val Ala Pro Gly Thr Phe Ser Asp
65   70   75   80

Leu Ile Lys Leu Lys Thr Leu Arg Leu Ser Arg Asn Lys Ile Thr His
                85   90   95

Leu Pro Gly Ala Leu Leu Asp Lys Met Val Leu Leu Glu Gln Leu Phe
            100   105   110

Leu Asp His Asn Ala Leu Arg Gly Ile Asp Gln Asn Met Phe Gln Lys
        115   120   125

Leu Val Asn Leu Gln Glu Leu Ala Leu Asn Gln Asn Gln Leu Asp Phe
    130   135   140

Leu Pro Ala Ser Leu Phe Thr Asn Leu Glu Asn Leu Lys Leu Leu Asp
145   150   155   160

Leu Ser Gly Asn Asn Leu Thr His Leu Pro Lys Gly Leu Leu Gly Ala
                165   170   175

Gln Ala Lys Leu Glu Arg Leu Leu Leu His Ser Asn Arg Leu Val Ser
            180   185   190

Leu Asp Ser Gly Leu Leu Asn Ser Leu Gly Ala Leu Thr Glu Leu Gln
        195   200   205

Phe His Arg Asn His Ile Arg Ser Ile Ala Pro Gly Ala Phe Asp Arg
    210   215   220

Leu Pro Asn Leu Ser Ser Leu Thr Leu Ser Arg Asn His Leu Ala Phe
225   230   235   240

Leu Pro Ser Ala Leu Phe Leu His Ser His Asn Leu Thr Leu Leu Thr
                245   250   255

Leu Phe Glu Asn Pro Leu Ala Glu Leu Pro Gly Val Leu Phe Gly Glu
            260   265   270

Met Gly Gly Leu Gln Glu Leu Trp Leu Asn Arg Thr Gln Leu Arg Thr
        275   280   285

Leu Pro Ala Ala Ala Phe Arg Asn Leu Ser Arg Leu Arg Tyr Leu Gly
    290   295   300

Val Thr Leu Ser Pro Arg Leu Ser Ala Leu Pro Gln Gly Ala Phe Gln
305   310   315   320

Gly Leu Gly Glu Leu Gln Val Leu Ala Leu His Ser Asn Gly Leu Thr
                325   330   335

Ala Leu Pro Asp Gly Leu Leu Arg Gly Leu Gly Lys Leu Arg Gln Val
            340   345   350

Ser Leu Arg Arg Asn Arg Leu Arg Ala Leu Pro Arg Ala Leu Phe Arg
        355   360   365

Asn Leu Ser Ser Leu Glu Ser Val Gln Leu Asp His Asn Gln Leu Glu
    370   375   380

Thr Leu Pro Gly Asp Val Phe Gly Ala Leu Pro Arg Leu Thr Glu Val
385   390   395   400

Leu Leu Gly His Asn Ser Trp Arg Cys Asp Cys Gly Leu Gly Pro Phe
                405   410   415

Leu Gly Trp Leu Arg Gln His Leu Gly Leu Val Gly Gly Glu Glu Pro
            420   425   430

Pro Arg Cys Ala Gly Pro Gly Ala His Ala Gly Leu Pro Leu Trp Ala
        435   440   445

Leu Pro Gly Gly Asp Ala Glu Cys Pro Gly Pro Arg Gly Pro Pro Pro
    450   455   460

Arg Pro Ala Ala Asp Ser Ser Ser Glu Ala Pro Val His Pro Ala Leu
465   470   475   480

Ala Pro Asn Ser Ser Glu Pro Trp Val Trp Ala Gln Pro Val Thr Thr
485 490 495

Gly Lys Gly Gln Asp His Ser Pro Phe Trp Gly Phe Tyr Phe Leu Leu
500 505 510

Leu Ala Val Gln Ala Met Ile Thr Val Ile Ile Val Phe Ala Met Ile
515 520 525

Lys Ile Gly Gln Leu Phe Arg Lys Leu Ile Arg Glu Arg Ala Leu Gly
530 535 540

<210> 4
<211> 527
<212> PRT
<213> Artificial Sequence

<220>
<223> Soluble GPV with C-terminal polyhistidine tag

<400> 4
Gln Pro Phe Pro Cys Pro Pro Ala Cys Lys Cys Val Phe Arg Asp Ala
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Ala Gln Cys Ser Gly Gly Asp Val Ala Arg Ile Ser Ala Leu Gly Leu
20 25 30

Pro Thr Asn Leu Thr His Ile Leu Leu Phe Gly Met Gly Arg Gly Val
35 40 45

Leu Gln Ser Gln Ser Phe Ser Gly Met Thr Val Leu Gln Arg Leu Met
50 55 60

Ile Ser Asp Ser His Ile Ser Ala Val Ala Pro Gly Thr Phe Ser Asp
65 70 75 80

Leu Ile Lys Leu Lys Thr Leu Arg Leu Ser Arg Asn Lys Ile Thr His
85 90 95

Leu Pro Gly Ala Leu Leu Asp Lys Met Val Leu Leu Glu Gln Leu Phe
100 105 110

Leu Asp His Asn Ala Leu Arg Gly Ile Asp Gln Asn Met Phe Gln Lys
115 120 125

Leu Val Asn Leu Gln Glu Leu Ala Leu Asn Gln Asn Gln Leu Asp Phe
130 135 140

Leu Pro Ala Ser Leu Phe Thr Asn Leu Glu Asn Leu Lys Leu Leu Asp
145 150 155 160

Leu Ser Gly Asn Asn Leu Thr His Leu Pro Lys Gly Leu Leu Gly Ala
165 170 175

Gln Ala Lys Leu Glu Arg Leu Leu Leu His Ser Asn Arg Leu Val Ser

180 185 190

Leu Asp Ser Gly Leu Leu Asn Ser Leu Gly Ala Leu Thr Glu Leu Gln
195 200 205

Phe His Arg Asn His Ile Arg Ser Ile Ala Pro Gly Ala Phe Asp Arg
210 215 220

Leu Pro Asn Leu Ser Ser Leu Thr Leu Ser Arg Asn His Leu Ala Phe
225 230 235 240

Leu Pro Ser Ala Leu Phe Leu His Ser His Asn Leu Thr Leu Leu Thr
245 250 255

Leu Phe Glu Asn Pro Leu Ala Glu Leu Pro Gly Val Leu Phe Gly Glu
260 265 270

Met Gly Gly Leu Gln Glu Leu Trp Leu Asn Arg Thr Gln Leu Arg Thr
275 280 285

Leu Pro Ala Ala Ala Phe Arg Asn Leu Ser Arg Leu Arg Tyr Leu Gly
290 295 300

Val Thr Leu Ser Pro Arg Leu Ser Ala Leu Pro Gln Gly Ala Phe Gln
305 310 315 320

Gly Leu Gly Glu Leu Gln Val Leu Ala Leu His Ser Asn Gly Leu Thr
325 330 335

Ala Leu Pro Asp Gly Leu Leu Arg Gly Leu Gly Lys Leu Arg Gln Val
340 345 350

Ser Leu Arg Arg Asn Arg Leu Arg Ala Leu Pro Arg Ala Leu Phe Arg
355 360 365

Asn Leu Ser Ser Leu Glu Ser Val Gln Leu Asp His Asn Gln Leu Glu
370 375 380

Thr Leu Pro Gly Asp Val Phe Gly Ala Leu Pro Arg Leu Thr Glu Val
385 390 395 400

Leu Leu Gly His Asn Ser Trp Arg Cys Asp Cys Gly Leu Gly Pro Phe
405 410 415

Leu Gly Trp Leu Arg Gln His Leu Gly Leu Val Gly Gly Glu Glu Pro
420 425 430

Pro Arg Cys Ala Gly Pro Gly Ala His Ala Gly Leu Pro Leu Trp Ala
435 440 445

Leu Pro Gly Gly Asp Ala Glu Cys Pro Gly Pro Arg Gly Pro Pro Pro
450 455 460

Arg Pro Ala Ala Asp Ser Ser Ser Glu Ala Pro Val His Pro Ala Leu
465 470 475 480

Ala Pro Asn Ser Ser Glu Pro Trp Val Trp Ala Gln Pro Val Thr Thr

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                485                 490                 495

Gly Lys Gly Gln Asp His Ser Pro Phe Trp Gly Phe Tyr Phe Leu Leu
            500                 505                 510

Leu Ala Val Gln Ala His His His His His His His His His His
        515                 520                 525
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<210> 5

<211> 2215

<212> DNA

<213> Mus musculus

<220>

<221> CDS

<222> (30)..(1733)

<400> 5

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                                Met Leu Arg Ser Ala Leu Leu Ser
                                1               5

gcg gtg ctc gca ctc ttg cgt gcc caa cct ttt ccc tgc ccc aaa acc     101
Ala Val Leu Ala Leu Leu Arg Ala Gln Pro Phe Pro Cys Pro Lys Thr
    10                  15                  20

tgc aag tgt gtg gtc cgc gat gcc gcg cag tgc tcg ggc ggc agc gtg     149
Cys Lys Cys Val Val Arg Asp Ala Ala Gln Cys Ser Gly Gly Ser Val
25                  30                  35                  40

gct cac atc gct gag cta ggt ctg cct acg aac ctc aca cac atc ctg     197
Ala His Ile Ala Glu Leu Gly Leu Pro Thr Asn Leu Thr His Ile Leu
                45                  50                  55

ctc ttc cga atg gac cag ggc ata ttg cgg aac cac agc ttc agc ggc     245
Leu Phe Arg Met Asp Gln Gly Ile Leu Arg Asn His Ser Phe Ser Gly
            60                  65                  70

atg aca gtc ctt cag cgc ctg atg ctc tca gat agc cac att tcc gcc     293
Met Thr Val Leu Gln Arg Leu Met Leu Ser Asp Ser His Ile Ser Ala
        75                  80                  85

atc gac ccc ggc acc ttc aat gac ctg gta aaa ctg aaa acc ctc agg     341
Ile Asp Pro Gly Thr Phe Asn Asp Leu Val Lys Leu Lys Thr Leu Arg
    90                  95                  100

ttg acg cgc aac aaa atc tct cgt ctt cca cgt gcg atc ctg gat aag     389
Leu Thr Arg Asn Lys Ile Ser Arg Leu Pro Arg Ala Ile Leu Asp Lys
105                 110                 115                 120

atg gta ctc ttg gaa cag ctg ttc ttg gac cac aat gca cta agg gac     437
Met Val Leu Leu Glu Gln Leu Phe Leu Asp His Asn Ala Leu Arg Asp
                125                 130                 135

ctt gat caa aac ctg ttt cag caa ctg cgt aac ctt cag gag ctc ggt     485
Leu Asp Gln Asn Leu Phe Gln Gln Leu Arg Asn Leu Gln Glu Leu Gly
            140                 145                 150

ttg aac cag aat cag ctc tct ttt ctt cct gct aac ctt ttc tcg agc     533
Leu Asn Gln Asn Gln Leu Ser Phe Leu Pro Ala Asn Leu Phe Ser Ser
        155                 160                 165

ctg aga gaa ctg aag ttg ttg gat tta tcg cga aac aac ctg acc cac     581
Leu Arg Glu Leu Lys Leu Leu Asp Leu Ser Arg Asn Asn Leu Thr His
    170                 175                 180
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ctg ccc aag gga ctg ctt ggg gct caa gtt aag ctt gag aaa ctg ctg 629
Leu Pro Lys Gly Leu Leu Gly Ala Gln Val Lys Leu Glu Lys Leu Leu
185 190 195 200

ctc tat tca aac cag ctc acg tct gtg gat tcg ggg ctg ctg agc aac 677
Leu Tyr Ser Asn Gln Leu Thr Ser Val Asp Ser Gly Leu Leu Ser Asn
205 210 215

ctg ggc gcc ctg act gag ctg cgg ctg gag cgg aat cac ctc cgc tcc 725
Leu Gly Ala Leu Thr Glu Leu Arg Leu Glu Arg Asn His Leu Arg Ser
220 225 230

gta gcc ccg ggt gcc ttc gac cgc ctc gga aac ctg agc tcc ttg act 773
Val Ala Pro Gly Ala Phe Asp Arg Leu Gly Asn Leu Ser Ser Leu Thr
235 240 245

cta tcc gga aac ctc ctg gag tct ctg ccg ccc gcg ctc ttc ctt cac 821
Leu Ser Gly Asn Leu Leu Glu Ser Leu Pro Pro Ala Leu Phe Leu His
250 255 260

gtg agc agc gtg tct cgg ctg act ctg ttc gag aac ccc ctg gag gag 869
Val Ser Ser Val Ser Arg Leu Thr Leu Phe Glu Asn Pro Leu Glu Glu
265 270 275 280

ctc ccg gac gtg ttg ttc ggg gag atg gcc ggc ctg cgg gag ctg tgg 917
Leu Pro Asp Val Leu Phe Gly Glu Met Ala Gly Leu Arg Glu Leu Trp
285 290 295

ctg aac ggc acc cac ctg agc acg ctg ccc gcc gct gcc ttc cgc aac 965
Leu Asn Gly Thr His Leu Ser Thr Leu Pro Ala Ala Ala Phe Arg Asn
300 305 310

ctg agc ggc ttg cag acg ctg ggg ctg acg cgg aac ccg cgc ctg agc 1013
Leu Ser Gly Leu Gln Thr Leu Gly Leu Thr Arg Asn Pro Arg Leu Ser

315 320 325

gcg ctc ccg cgc ggc gtg ttc cag ggc cta cgg gag ctg cgc gtg ctc 1061
Ala Leu Pro Arg Gly Val Phe Gln Gly Leu Arg Glu Leu Arg Val Leu
330 335 340

gcg ctg cac acc aac gcc ctg gcg gag ctg cgg gac gac gcg ctg cgc 1109
Ala Leu His Thr Asn Ala Leu Ala Glu Leu Arg Asp Asp Ala Leu Arg
345 350 355 360

ggc ctc ggg cac ctg cgc cag gtg tcg ctg cgc cac aac cgg ctg cgg 1157
Gly Leu Gly His Leu Arg Gln Val Ser Leu Arg His Asn Arg Leu Arg
365 370 375

gcc ctg ccc cgc acg ctc ttc cgc aac ctc agc agc ctc gag agc gtg 1205
Ala Leu Pro Arg Thr Leu Phe Arg Asn Leu Ser Ser Leu Glu Ser Val
380 385 390

cag cta gag cac aac cag ctg gag acg ctg cca gga gac gtg ttc gcg 1253
Gln Leu Glu His Asn Gln Leu Glu Thr Leu Pro Gly Asp Val Phe Ala
395 400 405

gct ctg ccc cag ctg acc cag gtc ctg ctg ggt cac aac ccc tgg ctc 1301
Ala Leu Pro Gln Leu Thr Gln Val Leu Leu Gly His Asn Pro Trp Leu
410 415 420

tgc gac tgt ggc ctg tgg ccc ttc ctc cag tgg ctg cgg cat cac ccg 1349
Cys Asp Cys Gly Leu Trp Pro Phe Leu Gln Trp Leu Arg His His Pro
425 430 435 440

gac atc ctg ggc cga gac gag ccc ccg cag tgc cgt ggc ccg gag cca 1397
Asp Ile Leu Gly Arg Asp Glu Pro Pro Gln Cys Arg Gly Pro Glu Pro
445 450 455

cgc gcc agc ctg tcg ttc tgg gag ctg ctg cag ggt gac ccg tgg tgc 1445
Arg Ala Ser Leu Ser Phe Trp Glu Leu Leu Gln Gly Asp Pro Trp Cys
460 465 470

ccg gat cct cgc agc ctg cct ctc gac cct cca acc gaa aat gct ctg 1493
Pro Asp Pro Arg Ser Leu Pro Leu Asp Pro Pro Thr Glu Asn Ala Leu
475 480 485

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gaa gcc ccg gtt ccg tcc tgg ctg cct aac agc tgg cag tcc cag acg       1541
Glu Ala Pro Val Pro Ser Trp Leu Pro Asn Ser Trp Gln Ser Gln Thr
    490                 495                 500

tgg gcc cag ctg gtg gcc agg ggt gaa agt ccc aat aac agg ctc tac       1589
Trp Ala Gln Leu Val Ala Arg Gly Glu Ser Pro Asn Asn Arg Leu Tyr
505                 510                 515                 520

tgg ggt ctt tat att ctg ctt cta gta gcc cag gcc atc ata gcc gcg       1637
Trp Gly Leu Tyr Ile Leu Leu Leu Val Ala Gln Ala Ile Ile Ala Ala
                525                 530                 535

ttc atc gtg ttt gcc atg att aaa atc ggc cag ctg ttt cga aca tta       1685
Phe Ile Val Phe Ala Met Ile Lys Ile Gly Gln Leu Phe Arg Thr Leu
            540                 545                 550

atc aga gag aag ctc ttg tta gag gca atg gga aaa tcg tgt aac taa       1733
Ile Arg Glu Lys Leu Leu Leu Glu Ala Met Gly Lys Ser Cys Asn
        555                 560                 565

tgaaactgac cagagcattg tggacggggc cccaaggaga atgcagtcag gatgctggcg     1793

tgccattaca ctatttccca ggccttttct cctctcccgt gctcttagtg tctcttcttc     1853

tcccctctct tcagaagtag cttttgtaaa tcgctactgc tttctagcct ggcctgggtt     1913

acctcctctg ctgttagttt caaggggggct gagggtgggg gttcgacggg acttggctca    1973

tcaggtccaa ctgtgcagcg ctgggtgcct agtggagaga ggagcccttt cttggtttct     2033

gaatttgagg acacatcctg ccagtgggca agacctctcc gggacccagc aagggttgag     2093

taacatttgc tgaaggaaca ccggcttaaa acgaacccta ggtccaagag atgaaggctc     2153

ttcccaaaat aaaggtggag tgttcttgtc cctttacctg aaaggaaaaa aaaaaaaaaa     2213

aa                                                                    2215
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<210> 6
<211> 567
<212> PRT
<213> Mus musculus

<400> 6

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Met Leu Arg Ser Ala Leu Leu Ser Ala Val Leu Ala Leu Leu Arg Ala
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Gln Pro Phe Pro Cys Pro Lys Thr Cys Lys Cys Val Val Arg Asp Ala
            20                  25                  30

Ala Gln Cys Ser Gly Gly Ser Val Ala His Ile Ala Glu Leu Gly Leu
        35                  40                  45

Pro Thr Asn Leu Thr His Ile Leu Leu Phe Arg Met Asp Gln Gly Ile
    50                  55                  60

Leu Arg Asn His Ser Phe Ser Gly Met Thr Val Leu Gln Arg Leu Met
65                  70                  75                  80

Leu Ser Asp Ser His Ile Ser Ala Ile Asp Pro Gly Thr Phe Asn Asp
                85                  90                  95

Leu Val Lys Leu Lys Thr Leu Arg Leu Thr Arg Asn Lys Ile Ser Arg
            100                 105                 110
```

Leu Pro Arg Ala Ile Leu Asp Lys Met Val Leu Leu Glu Gln Leu Phe
115 120 125

Leu Asp His Asn Ala Leu Arg Asp Leu Asp Gln Asn Leu Phe Gln Gln
130 135 140

Leu Arg Asn Leu Gln Glu Leu Gly Leu Asn Gln Asn Gln Leu Ser Phe
145 150 155 160

Leu Pro Ala Asn Leu Phe Ser Ser Leu Arg Glu Leu Lys Leu Leu Asp
165 170 175

Leu Ser Arg Asn Asn Leu Thr His Leu Pro Lys Gly Leu Leu Gly Ala
180 185 190

Gln Val Lys Leu Glu Lys Leu Leu Leu Tyr Ser Asn Gln Leu Thr Ser
195 200 205

Val Asp Ser Gly Leu Leu Ser Asn Leu Gly Ala Leu Thr Glu Leu Arg
210 215 220

Leu Glu Arg Asn His Leu Arg Ser Val Ala Pro Gly Ala Phe Asp Arg
225 230 235 240

Leu Gly Asn Leu Ser Ser Leu Thr Leu Ser Gly Asn Leu Leu Glu Ser
245 250 255

Leu Pro Pro Ala Leu Phe Leu His Val Ser Ser Val Ser Arg Leu Thr
260 265 270

Leu Phe Glu Asn Pro Leu Glu Glu Leu Pro Asp Val Leu Phe Gly Glu
275 280 285

Met Ala Gly Leu Arg Glu Leu Trp Leu Asn Gly Thr His Leu Ser Thr
290 295 300

Leu Pro Ala Ala Ala Phe Arg Asn Leu Ser Gly Leu Gln Thr Leu Gly
305 310 315 320

Leu Thr Arg Asn Pro Arg Leu Ser Ala Leu Pro Arg Gly Val Phe Gln
325 330 335

Gly Leu Arg Glu Leu Arg Val Leu Ala Leu His Thr Asn Ala Leu Ala
340 345 350

Glu Leu Arg Asp Asp Ala Leu Arg Gly Leu Gly His Leu Arg Gln Val
355 360 365

Ser Leu Arg His Asn Arg Leu Arg Ala Leu Pro Arg Thr Leu Phe Arg
370 375 380

Asn Leu Ser Ser Leu Glu Ser Val Gln Leu Glu His Asn Gln Leu Glu
385 390 395 400

Thr Leu Pro Gly Asp Val Phe Ala Ala Leu Pro Gln Leu Thr Gln Val

405 410 415

Leu Leu Gly His Asn Pro Trp Leu Cys Asp Cys Gly Leu Trp Pro Phe
420 425 430

Leu Gln Trp Leu Arg His His Pro Asp Ile Leu Gly Arg Asp Glu Pro
435 440 445

Pro Gln Cys Arg Gly Pro Glu Pro Arg Ala Ser Leu Ser Phe Trp Glu
450 455 460

Leu Leu Gln Gly Asp Pro Trp Cys Pro Asp Pro Arg Ser Leu Pro Leu
465 470 475 480

Asp Pro Pro Thr Glu Asn Ala Leu Glu Ala Pro Val Pro Ser Trp Leu
485 490 495

Pro Asn Ser Trp Gln Ser Gln Thr Trp Ala Gln Leu Val Ala Arg Gly
500 505 510

Glu Ser Pro Asn Asn Arg Leu Tyr Trp Gly Leu Tyr Ile Leu Leu Leu
515 520 525

Val Ala Gln Ala Ile Ile Ala Ala Phe Ile Val Phe Ala Met Ile Lys
530 535 540

Ile Gly Gln Leu Phe Arg Thr Leu Ile Arg Glu Lys Leu Leu Leu Glu
545 550 555 560

Ala Met Gly Lys Ser Cys Asn
565

<210> 7
<211> 551
<212> PRT
<213> Mus musculus

<400> 7
Gln Pro Phe Pro Cys Pro Lys Thr Cys Lys Cys Val Val Arg Asp Ala
1 5 10 15

Ala Gln Cys Ser Gly Gly Ser Val Ala His Ile Ala Glu Leu Gly Leu
20 25 30

Pro Thr Asn Leu Thr His Ile Leu Leu Phe Arg Met Asp Gln Gly Ile
35 40 45

Leu Arg Asn His Ser Phe Ser Gly Met Thr Val Leu Gln Arg Leu Met

50 55 60

Leu Ser Asp Ser His Ile Ser Ala Ile Asp Pro Gly Thr Phe Asn Asp
65 70 75 80

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Leu Val Lys Leu Lys Thr Leu Arg Leu Thr Arg Asn Lys Ile Ser Arg
                85                  90                  95

Leu Pro Arg Ala Ile Leu Asp Lys Met Val Leu Leu Glu Gln Leu Phe
            100                 105                 110

Leu Asp His Asn Ala Leu Arg Asp Leu Asp Gln Asn Leu Phe Gln Gln
        115                 120                 125

Leu Arg Asn Leu Gln Glu Leu Gly Leu Asn Gln Asn Gln Leu Ser Phe
    130                 135                 140

Leu Pro Ala Asn Leu Phe Ser Ser Leu Arg Glu Leu Lys Leu Leu Asp
145                 150                 155                 160

Leu Ser Arg Asn Asn Leu Thr His Leu Pro Lys Gly Leu Leu Gly Ala
                165                 170                 175

Gln Val Lys Leu Glu Lys Leu Leu Leu Tyr Ser Asn Gln Leu Thr Ser
            180                 185                 190

Val Asp Ser Gly Leu Leu Ser Asn Leu Gly Ala Leu Thr Glu Leu Arg
        195                 200                 205

Leu Glu Arg Asn His Leu Arg Ser Val Ala Pro Gly Ala Phe Asp Arg
    210                 215                 220

Leu Gly Asn Leu Ser Ser Leu Thr Leu Ser Gly Asn Leu Leu Glu Ser
225                 230                 235                 240

Leu Pro Pro Ala Leu Phe Leu His Val Ser Ser Val Ser Arg Leu Thr
                245                 250                 255

Leu Phe Glu Asn Pro Leu Glu Glu Leu Pro Asp Val Leu Phe Gly Glu
            260                 265                 270

Met Ala Gly Leu Arg Glu Leu Trp Leu Asn Gly Thr His Leu Ser Thr
        275                 280                 285

Leu Pro Ala Ala Ala Phe Arg Asn Leu Ser Gly Leu Gln Thr Leu Gly
    290                 295                 300

Leu Thr Arg Asn Pro Arg Leu Ser Ala Leu Pro Arg Gly Val Phe Gln
305                 310                 315                 320

Gly Leu Arg Glu Leu Arg Val Leu Ala Leu His Thr Asn Ala Leu Ala
                325                 330                 335

Glu Leu Arg Asp Asp Ala Leu Arg Gly Leu Gly His Leu Arg Gln Val
            340                 345                 350

Ser Leu Arg His Asn Arg Leu Arg Ala Leu Pro Arg Thr Leu Phe Arg
        355                 360                 365

Asn Leu Ser Ser Leu Glu Ser Val Gln Leu Glu His Asn Gln Leu Glu
    370                 375                 380
```

Thr Leu Pro Gly Asp Val Phe Ala Ala Leu Pro Gln Leu Thr Gln Val
385 390 395 400

Leu Leu Gly His Asn Pro Trp Leu Cys Asp Cys Gly Leu Trp Pro Phe
405 410 415

Leu Gln Trp Leu Arg His His Pro Asp Ile Leu Gly Arg Asp Glu Pro
420 425 430

Pro Gln Cys Arg Gly Pro Glu Pro Arg Ala Ser Leu Ser Phe Trp Glu
435 440 445

Leu Leu Gln Gly Asp Pro Trp Cys Pro Asp Pro Arg Ser Leu Pro Leu
450 455 460

Asp Pro Pro Thr Glu Asn Ala Leu Glu Ala Pro Val Pro Ser Trp Leu
465 470 475 480

Pro Asn Ser Trp Gln Ser Gln Thr Trp Ala Gln Leu Val Ala Arg Gly
485 490 495

Glu Ser Pro Asn Asn Arg Leu Tyr Trp Gly Leu Tyr Ile Leu Leu Leu
500 505 510

Val Ala Gln Ala Ile Ile Ala Ala Phe Ile Val Phe Ala Met Ile Lys
515 520 525

Ile Gly Gln Leu Phe Arg Thr Leu Ile Arg Glu Lys Leu Leu Leu Glu
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Ala Met Gly Lys Ser Cys Asn
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35 40 45

Leu Arg Asn His Ser Phe Ser Gly Met Thr Val Leu Gln Arg Gln Met
50 55 60

Leu Ser Asn Ser His Ile Ser Ala Ile Asn Pro Gly Thr Phe Asn Asn

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Leu Ser Asp Ser His Ile Ser Ala Ile Asp Pro Gly Thr Phe Asn Asp
65                  70                  75                  80

Leu Val Lys Leu Lys Thr Leu Arg Leu Thr Arg Asn Lys Ile Ser Arg
                85                  90                  95

Leu Pro Arg Ala Ile Leu Asp Lys Met Val Leu Leu Glu Gln Leu Phe
            100                 105                 110

Leu Asp His Asn Ala Leu Arg Asp Leu Asp Gln Asn Leu Phe Gln Gln
        115                 120                 125

Leu Arg Asn Leu Gln Glu Leu Gly Leu Asn Gln Asn Gln Leu Ser Phe
    130                 135                 140

Leu Pro Ala Asn Leu Phe Ser Ser Leu Arg Glu Leu Lys Leu Leu Asp
145                 150                 155                 160

Leu Ser Arg Asn Asn Leu Thr His Leu Pro Lys Gly Leu Leu Gly Ala
                165                 170                 175

Gln Val Lys Leu Glu Lys Leu Leu Leu Tyr Ser Asn Gln Leu Thr Ser
            180                 185                 190

Val Asp Ser Gly Leu Leu Ser Asn Leu Gly Ala Leu Thr Glu Leu Arg
        195                 200                 205

Leu Glu Arg Asn His Leu Arg Ser Val Ala Pro Gly Ala Phe Asp Arg
    210                 215                 220

Leu Gly Asn Leu Ser Ser Leu Thr Leu Ser Gly Asn Leu Leu Glu Ser
225                 230                 235                 240

Leu Pro Pro Ala Leu Phe Leu His Val Ser Ser Val Ser Arg Leu Thr
                245                 250                 255

Leu Phe Glu Asn Pro Leu Glu Glu Leu Pro Asp Val Leu Phe Gly Glu
            260                 265                 270

Met Ala Gly Leu Arg Glu Leu Trp Leu Asn Gly Thr His Leu Ser Thr
        275                 280                 285

Leu Pro Ala Ala Ala Phe Arg Asn Leu Ser Gly Leu Gln Thr Leu Gly
    290                 295                 300

Leu Thr Arg Asn Pro Arg Leu Ser Ala Leu Pro Arg Gly Val Phe Gln
305                 310                 315                 320

Gly Leu Arg Glu Leu Arg Val Leu Gly Leu His Thr Asn Ala Leu Ala
                325                 330                 335

Glu Leu Arg Asp Asp Ala Leu Arg Gly Leu Gly His Leu Arg Gln Val
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Ser Leu Arg His Asn Arg Leu Arg Ala Leu Pro Arg Thr Leu Phe Arg
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Asn Leu Ser Ser Leu Glu Ser Val Gln Leu Glu His Asn Gln Leu Glu
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Thr Leu Pro Gly Asp Val Phe Ala Ala Leu Pro Gln Leu Thr Gln Val
385                 390                 395                 400

Leu Leu Gly His Asn Pro Trp Leu Cys Asp Cys Gly Leu Trp Arg Phe
                405                 410                 415

Leu Gln Trp Leu Arg His His Pro Asp Ile Leu Gly Arg Asp Glu Pro
            420                 425                 430

Pro Gln Cys Arg Gly Pro Glu Pro Arg Ala Ser Leu Ser Phe Trp Glu
        435                 440                 445

Leu Leu Gln Gly Asp Pro Trp Cys Pro Asp Pro Arg Ser Leu Pro Leu
    450                 455                 460

Asp Pro Pro Thr Glu Asn Ala Leu Glu Ala Pro Val Pro Ser Trp Leu
465                 470                 475                 480

Pro Asn Ser Trp Gln Ser Gln Thr Trp Ala Gln Leu Val Ala Arg Gly
                485                 490                 495

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Pro Thr Asn Leu Thr His Ile Leu Leu Phe Gly Met Gly Arg Gly Val
        35                  40                  45

Leu Gln Ser Gln Ser Phe Ser Gly Met Thr Val Leu Gln Arg Leu Met
    50                  55                  60

Ile Ser Asp Ser His Ile Ser Ala Val Ala Pro Gly Thr Phe Ser Asp
65                  70                  75                  80

Leu Ile Lys Leu Lys Thr Leu Arg Leu Ser Arg Asn Lys Ile Thr His
                85                  90                  95

Leu Pro Gly Ala Leu Leu Asp Lys Met Val Leu Leu Glu Gln Leu Phe
            100                 105                 110

Leu Asp His Asn Ala Leu Arg Gly Ile Asp Gln Asn Met Phe Gln Lys
        115                 120                 125

Leu Val Asn Leu Gln Glu Leu Ala Leu Asn Gln Asn Gln Leu Asp Phe
    130                 135                 140

Leu Pro Ala Ser Leu Phe Thr Asn Leu Glu Asn Leu Lys Leu Leu Asp
145                 150                 155                 160

Leu Ser Gly Asn Asn Leu Thr His Leu Pro Lys Gly Leu Leu Gly Ala
                165                 170                 175

Gln Ala Lys Leu Glu Arg Leu Leu Leu His Ser Asn Arg Leu Val Ser
            180                 185                 190

Leu Asp Ser Gly Leu Leu Asn Ser Leu Gly Ala Leu Thr Glu Leu Gln
        195                 200                 205

Phe His Arg Asn His Ile Arg Ser Ile Ala Pro Gly Ala Phe Asp Arg
    210                 215                 220

Leu Pro Asn Leu Ser Ser Leu Thr Leu Ser Arg Asn His Leu Ala Phe
225                 230                 235                 240

Leu Pro Ser Ala Leu Phe Leu His Ser His Asn Leu Thr Leu Leu Thr
                245                 250                 255

Leu Phe Glu Asn Pro Leu Ala Glu Leu Pro Gly Val Leu Phe Gly Glu
            260                 265                 270
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Met Gly Gly Leu Gln Glu Leu Trp Leu Asn Arg Thr Gln Leu Arg Thr
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Leu Pro Ala Ala Ala Phe Arg Asn Leu Ser Arg Leu Arg Tyr Leu Gly
290 295 300

Val Thr Leu Ser Pro Arg Leu Ser Ala Leu Pro Gln Gly Ala Phe Gln

305 310 315 320

Gly Leu Gly Glu Leu Gln Val Leu Ala Leu His Ser Asn Gly Leu Thr
325 330 335

Ala Leu Pro Asp Gly Leu Leu Arg Gly Leu Gly Lys Leu Arg Gln Val
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Ser Leu Arg Arg Asn Arg Leu Arg Ala Leu Pro Arg Ala Leu Phe Arg
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Asn Leu Ser Ser Leu Glu Ser Val Gln Leu Asp His Asn Gln Leu Glu
370 375 380

Thr Leu Pro Gly Asp Val Phe Gly Ala Leu Pro Arg Leu Thr Glu Val
385 390 395 400

Leu Leu Gly His Asn Ser Trp Arg Cys Asp Cys Gly Leu Gly Pro Phe
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Leu Gly Trp Leu Arg Gln His Leu Gly Leu Val Gly Gly Glu Glu Pro
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465 470 475 480

Gly Ser Gly Gly Ser Gly Gly Ser Gly Gly Ser Gly Ser Asp Ala His
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Lys Ser Glu Val Ala His Arg Phe Lys Asp Leu Gly Glu Glu Asn Phe
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Lys Ala Leu Val Leu Ile Ala Phe Ala Gln Tyr Leu Gln Gln Cys Pro
515 520 525

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Glu Cys Phe Leu Gln His Lys Asp Asp Asn Pro Asn Leu Pro Arg Leu
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Val Arg Pro Glu Val Asp Val Met Cys Thr Ala Phe His Asp Asn Glu
    610                 615                 620

Glu Thr Phe Leu Lys Lys Tyr Leu Tyr Glu Ile Ala Arg Arg His Pro
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Tyr Phe Tyr Ala Pro Glu Leu Leu Phe Phe Ala Lys Arg Tyr Lys Ala
                645                 650                 655

Ala Phe Thr Glu Cys Cys Gln Ala Ala Asp Lys Ala Ala Cys Leu Leu
            660                 665                 670

Pro Lys Leu Asp Glu Leu Arg Asp Glu Gly Lys Ala Ser Ser Ala Lys
        675                 680                 685

Gln Arg Leu Lys Cys Ala Ser Leu Gln Lys Phe Gly Glu Arg Ala Phe
    690                 695                 700

Lys Ala Trp Ala Val Ala Arg Leu Ser Gln Arg Phe Pro Lys Ala Glu
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Phe Ala Glu Val Ser Lys Leu Val Thr Asp Leu Thr Lys Val His Thr
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Glu Cys Cys His Gly Asp Leu Leu Glu Cys Ala Asp Asp Arg Ala Asp
            740                 745                 750

Leu Ala Lys Tyr Ile Cys Glu Asn Gln Asp Ser Ile Ser Ser Lys Leu
        755                 760                 765

Lys Glu Cys Cys Glu Lys Pro Leu Leu Glu Lys Ser His Cys Ile Ala
    770                 775                 780

Glu Val Glu Asn Asp Glu Met Pro Ala Asp Leu Pro Ser Leu Ala Ala
785                 790                 795                 800

Asp Phe Val Glu Ser Lys Asp Val Cys Lys Asn Tyr Ala Glu Ala Lys
                805                 810                 815

Asp Val Phe Leu Gly Met Phe Leu Tyr Glu Tyr Ala Arg Arg His Pro
            820                 825                 830

Asp Tyr Ser Val Val Leu Leu Leu Arg Leu Ala Lys Thr Tyr Glu Thr
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Thr Leu Glu Lys Cys Cys Ala Ala Ala Asp Pro His Glu Cys Tyr Ala
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865 870 875 880

Ile Lys Gln Asn Cys Glu Leu Phe Glu Gln Leu Gly Glu Tyr Lys Phe
885 890 895

Gln Asn Ala Leu Leu Val Arg Tyr Thr Lys Lys Val Pro Gln Val Ser
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Thr Pro Thr Leu Val Glu Val Ser Arg Asn Leu Gly Lys Val Gly Ser
915 920 925

Lys Cys Cys Lys His Pro Glu Ala Lys Arg Met Pro Cys Ala Glu Asp
930 935 940

Tyr Leu Ser Val Val Leu Asn Gln Leu Cys Val Leu His Glu Lys Thr
945 950 955 960

Pro Val Ser Asp Arg Val Thr Lys Cys Cys Thr Glu Ser Leu Val Asn
965 970 975

Arg Arg Pro Cys Phe Ser Ala Leu Glu Val Asp Glu Thr Tyr Val Pro
980 985 990

Lys Glu Phe Asn Ala Glu Thr Phe Thr Phe His Ala Asp Ile Cys Thr
995 1000 1005

Leu Ser Glu Lys Glu Arg Gln Ile Lys Lys Gln Thr Ala Leu Val
1010 1015 1020

Glu Leu Val Lys His Lys Pro Lys Ala Thr Lys Glu Gln Leu Lys
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Leu Gln Ser Gln Ser Phe Ser Gly Met Thr Val Leu Gln Arg Leu Met
50 55 60

Ile Ser Asp Ser His Ile Ser Ala Val Ala Pro Gly Thr Phe Ser Asp
65 70 75 80

Leu Ile Lys Leu Lys Thr Leu Arg Leu Ser Arg Asn Lys Ile Thr His
85 90 95

Leu Pro Gly Ala Leu Leu Asp Lys Met Val Leu Leu Glu Gln Leu Phe
100 105 110

Leu Asp His Asn Ala Leu Arg Gly Ile Asp Gln Asn Met Phe Gln Lys
115 120 125

Leu Val Asn Leu Gln Glu Leu Ala Leu Asn Gln Asn Gln Leu Asp Phe
130 135 140

Leu Pro Ala Ser Leu Phe Thr Asn Leu Glu Asn Leu Lys Leu Leu Asp
145 150 155 160

Leu Ser Gly Asn Asn Leu Thr His Leu Pro Lys Gly Leu Leu Gly Ala
165 170 175

Gln Ala Lys Leu Glu Arg Leu Leu Leu His Ser Asn Arg Leu Val Ser
180 185 190

Leu Asp Ser Gly Leu Leu Asn Ser Leu Gly Ala Leu Thr Glu Leu Gln
195 200 205

Phe His Arg Asn His Ile Arg Ser Ile Ala Pro Gly Ala Phe Asp Arg
210 215 220

Leu Pro Asn Leu Ser Ser Leu Thr Leu Ser Arg Asn His Leu Ala Phe
225 230 235 240

Leu Pro Ser Ala Leu Phe Leu His Ser His Asn Leu Thr Leu Leu Thr
245 250 255

Leu Phe Glu Asn Pro Leu Ala Glu Leu Pro Gly Val Leu Phe Gly Glu
260 265 270

Met Gly Gly Leu Gln Glu Leu Trp Leu Asn Arg Thr Gln Leu Arg Thr
275 280 285

Leu Pro Ala Ala Ala Phe Arg Asn Leu Ser Arg Leu Arg Tyr Leu Gly
290 295 300

Val Thr Leu Ser Pro Arg Leu Ser Ala Leu Pro Gln Gly Ala Phe Gln
305 310 315 320

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                325                 330                 335

Ala Leu Pro Asp Gly Leu Leu Arg Gly Leu Gly Lys Leu Arg Gln Val
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Ser Leu Arg Arg Asn Arg Leu Arg Ala Leu Pro Arg Ala Leu Phe Arg
        355                 360                 365

Asn Leu Ser Ser Leu Glu Ser Val Gln Leu Asp His Asn Gln Leu Glu
    370                 375                 380

Thr Leu Pro Gly Asp Val Phe Gly Ala Leu Pro Arg Leu Thr Glu Val
385                 390                 395                 400

Leu Leu Gly His Asn Ser Trp Arg Cys Asp Cys Gly Leu Gly Pro Phe
                405                 410                 415

Leu Gly Trp Leu Arg Gln His Leu Gly Leu Val Gly Gly Glu Glu Pro
            420                 425                 430

Pro Arg Cys Ala Gly Pro Gly Ala His Ala Gly Leu Pro Leu Trp Ala
        435                 440                 445

Leu Pro Gly Gly Asp Ala Glu Cys Pro Gly Pro Arg
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PATENTKRAV

1. Et opløseligt polypeptid omfattende et modificeret glycoprotein V (GPV), der mangler et funktionelt transmembrane domæne til anvendelse i behandlingen og/eller forebyggelse af en trombotisk sygdom hos et individ, nævnte behandling eller forebyggelse omfatter administration til individet af en effektiv mængde af nævnte opløselige polypeptid,
hvor det opløselige polypeptid ikke er i stand til at blive integreret i en cellemembran via nævnte transmembrane domæne,
hvor udtrykket GPV betegner et protein med en sekvensidentitet på mindst 70% med aminosyresekvensen som vist i SEQ ID NO: 3.

2. Opløseligt polypeptid til anvendelse ifølge krav 1, hvor den trombotiske sygdom er udvalgt blandt gruppen bestående af tromboinflammatoriske tilstande, venøs trombose, arteriel thrombose, kapillær trombose, vena porta trombose, renal venetrombose, jugular vene-trombose, cerebral trombose venøs sinustrombose, trombedannelse under eller efter at bringe blod i kontakt med en kunstig overflade, især ekstrakorporeal membranoxygenation (ECMO), åreforkalkning, arthritis, koagulopati, dyb venøs trombose (DVT), dessimeneret intravaskulær koagulopati (DIC), en kronisk eller akut tromboemboli, lungetromboemboli, Budd-Chiari syndrom, Paget-Schroetter sygdom, slagtilfælde og myokardieinfraktion.

3. Opløseligt polypeptid til anvendelse ifølge et hvilket som helst af de foregående krav, hvor nævnte modificerede GPV er en trunkeret GPV.

4. Opløseligt polypeptid til anvendelse ifølge et hvilket som helst af de foregående krav, hvor nævnte modificerede GPV består af et fragment af det ekstracellulære domæne af en oprindelig GPV, hvor nævnte fragment mangler det transmembrane domænet af nævnte oprindelige GPV.

5. Opløseligt polypeptid til anvendelse ifølge krav 4, hvor nævnte oprindelige GPV består af aminosyresekvensen som vist i SEQ ID NO: 3.

6. Opløseligt polypeptid til anvendelse ifølge et hvilket som helst af de foregående krav, hvor nævnte opløselige polypeptid er et ikke-naturligt forekommende polypeptid.

7. Opløseligt polypeptid til anvendelse ifølge krav 6, yderligere omfattende en halveringstidsforlængende del.

8. Opløseligt polypeptid til anvendelse ifølge krav 7, hvor nævnte halveringstidsforlængende del er konjugeret til nævnte modificerede GPV.

9. Opløseligt polypeptid til anvendelse ifølge krav 8, hvor nævnte halveringstidsforlængende del er udvalgt blandt gruppen bestående af hydroxyethylstivelse (HES), polyethylenglycol (PEG), polysialsyrer (PSA'er) og albuminbindende ligander, f.eks. fedtsyrekæder.

10. Opløseligt polypeptid til anvendelse ifølge krav 7, hvor nævnte halveringstidsforlængende del er en heterolog aminosyresekvens fusioneret til nævnte modificerede GPV, enten direkte eller via en linker.

11. Opløseligt polypeptid til anvendelse ifølge krav 10, hvor den halveringstidsforlængende heterolog aminosyresekvens, omfatter eller består af et polypeptid udvalgt blandt gruppen bestående af albumin og et fragment deraf med en længde på mindst 100 aminosyrer, immunoglobulin konstante regioner og fragmenter deraf, i især Fc-fragmentet, transferrin og fragmenter deraf, det C-terminale peptid af humant choriongonadotropin, solvatiserede tilfældige kæder med stort hydrodynamisk volumen (XTEN), homo-aminosyre gentagelser (HAP), prolin-alanineserin gentagelser (PAS), afamin, alfa-fetoprotein, vitamin D-bindingsprotein, polypeptider, der er i stand til at binde under fysiologiske betingelser til albumin eller immunglobulin konstante regioner og kombinationer deraf.

12. Opløseligt polypeptid til anvendelse ifølge et hvilket som helst af de foregående krav, hvor nævnte opløselige polypeptid kan opnås ved rekombinant ekspression i pattedyrceller.

13. Opløseligt polypeptid til anvendelse ifølge et hvilket som helst af de foregående krav, hvor nævnte behandling og/eller forebyggelse yderligere omfatter administration af en trombocytfunktionshæmmende- eller et antikoagulantlægemiddel til nævnte individ.

14. Farmaceutisk sammensætning omfattende et opløseligt polypeptid som defineret i et hvilket som helst af kravene 1 til 12 og en farmaceutisk acceptabel excipiens.

15. Farmaceutisk sammensætning ifølge krav 14, hvor det opløselige polypeptid ikke består af aminosyresekvensen som vist i SEQ ID NO: 10.

16. Fremgangsmåde til fremstilling af det opløselige polypeptid ifølge et hvilket som helst af kravene 1 til 12, omfattende ekspression af en nukleinsyre kodende for det opløselige polypeptid som defineret i et hvilket som helst af kravene 1 til 12 i en pattedyrscelle, og udvinding af det opløselige polypeptid fra kulturmediet.

17. Opløseligt polypeptid ifølge krav 6, hvor det modificerede glycoprotein V (GPV) er et ikke-naturligt forekommende opløseligt GPV.

18. Farmaceutisk kit omfattende (i) et opløseligt polypeptid ifølge et hvilket som helst af kravene 1 til 12, og (ii) et trombocytfunktionshæmmende- eller et antikoagulantlægemiddel andet end det opløselige polypeptid.

DRAWINGS

Figure 1

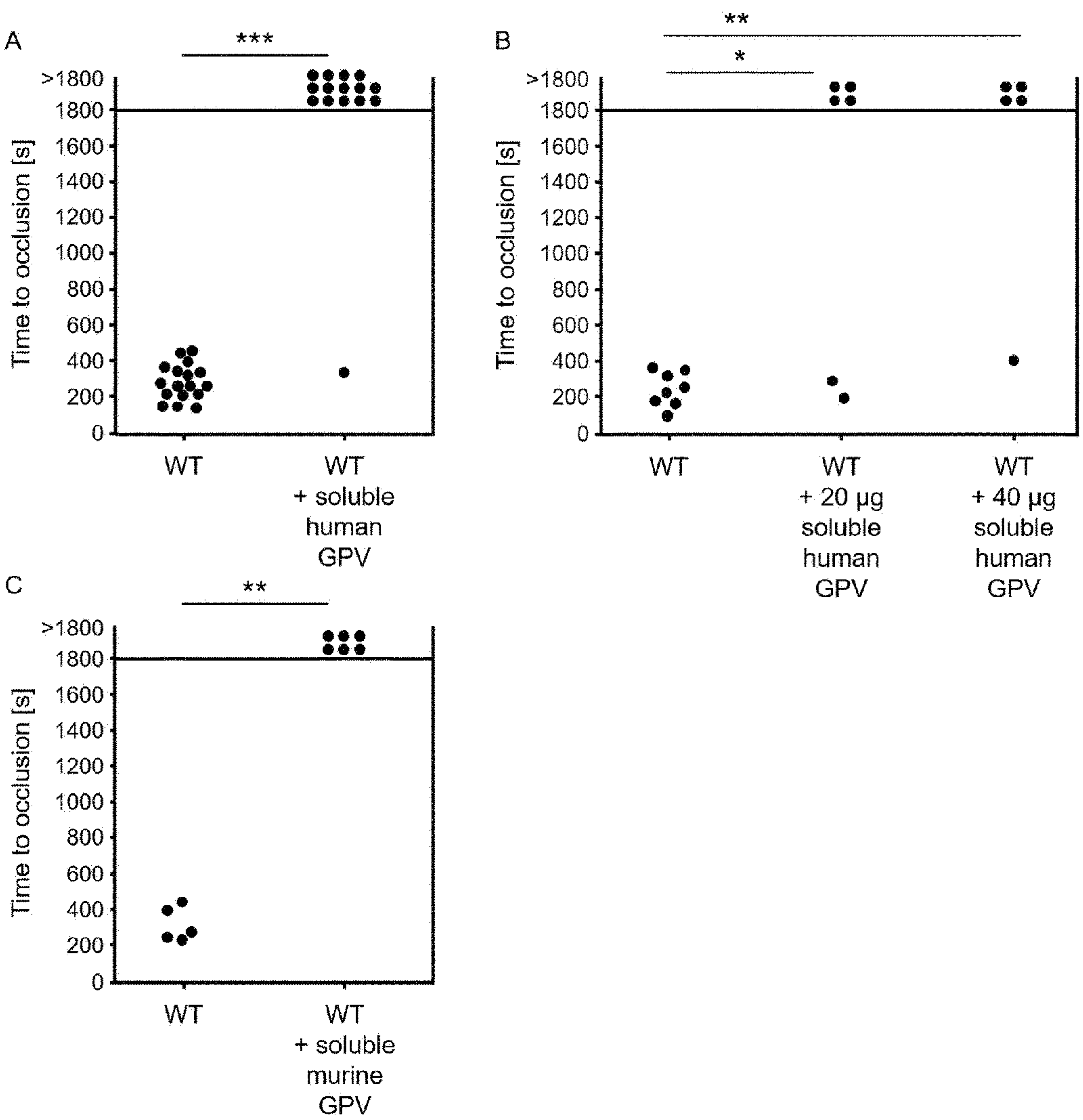


Figure 2

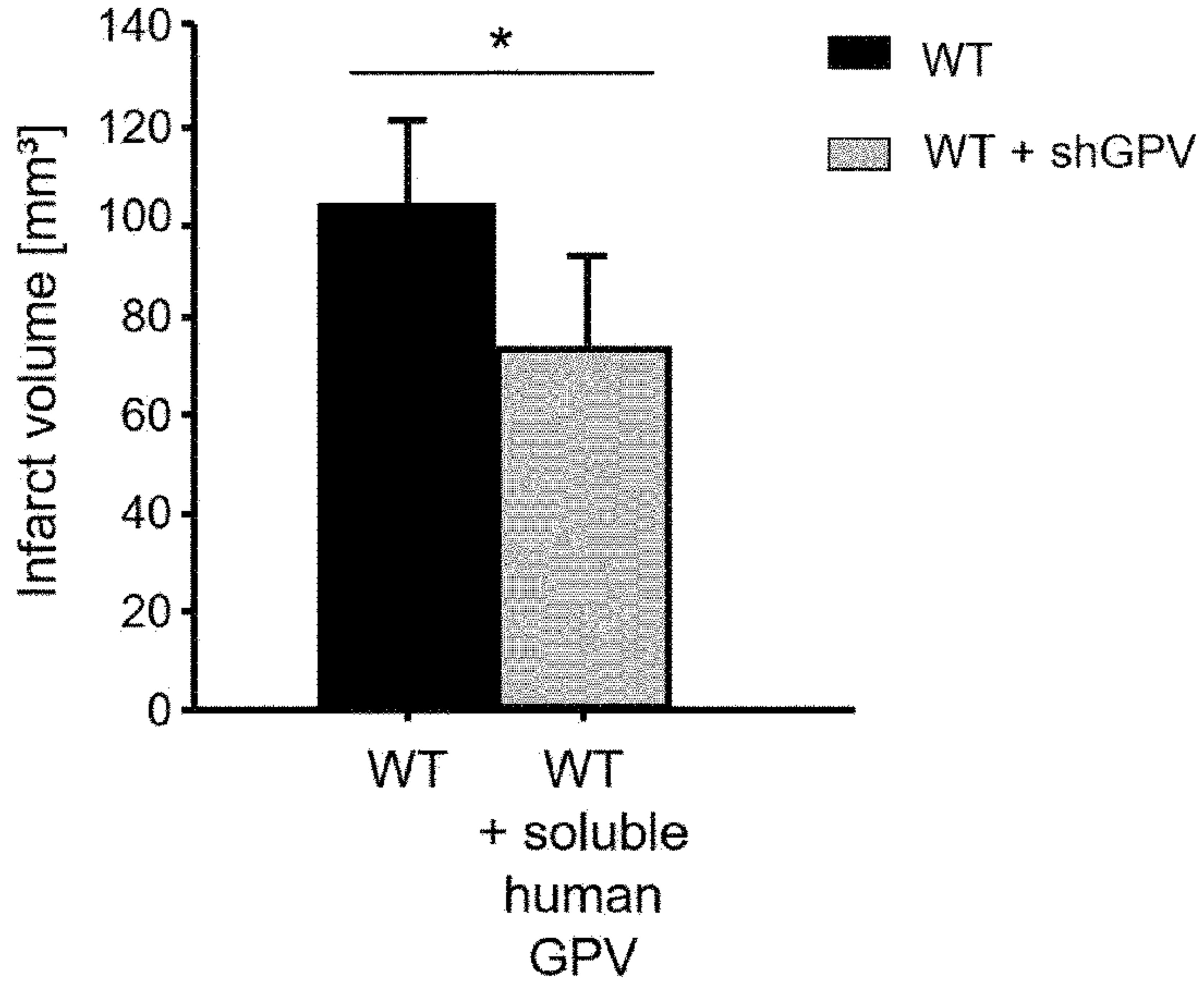


Figure 3

Tail bleeding time: WT + soluble human GPV

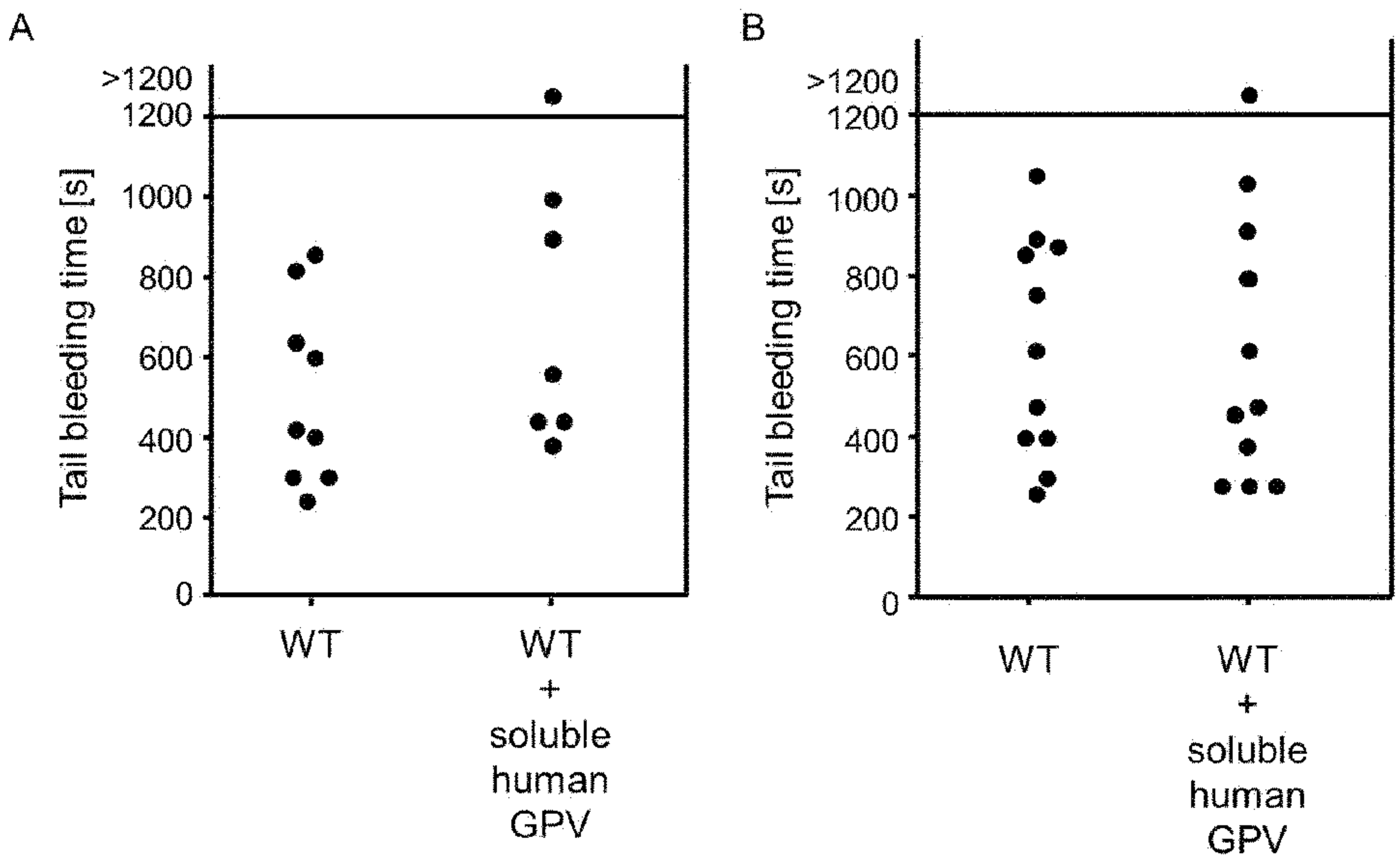
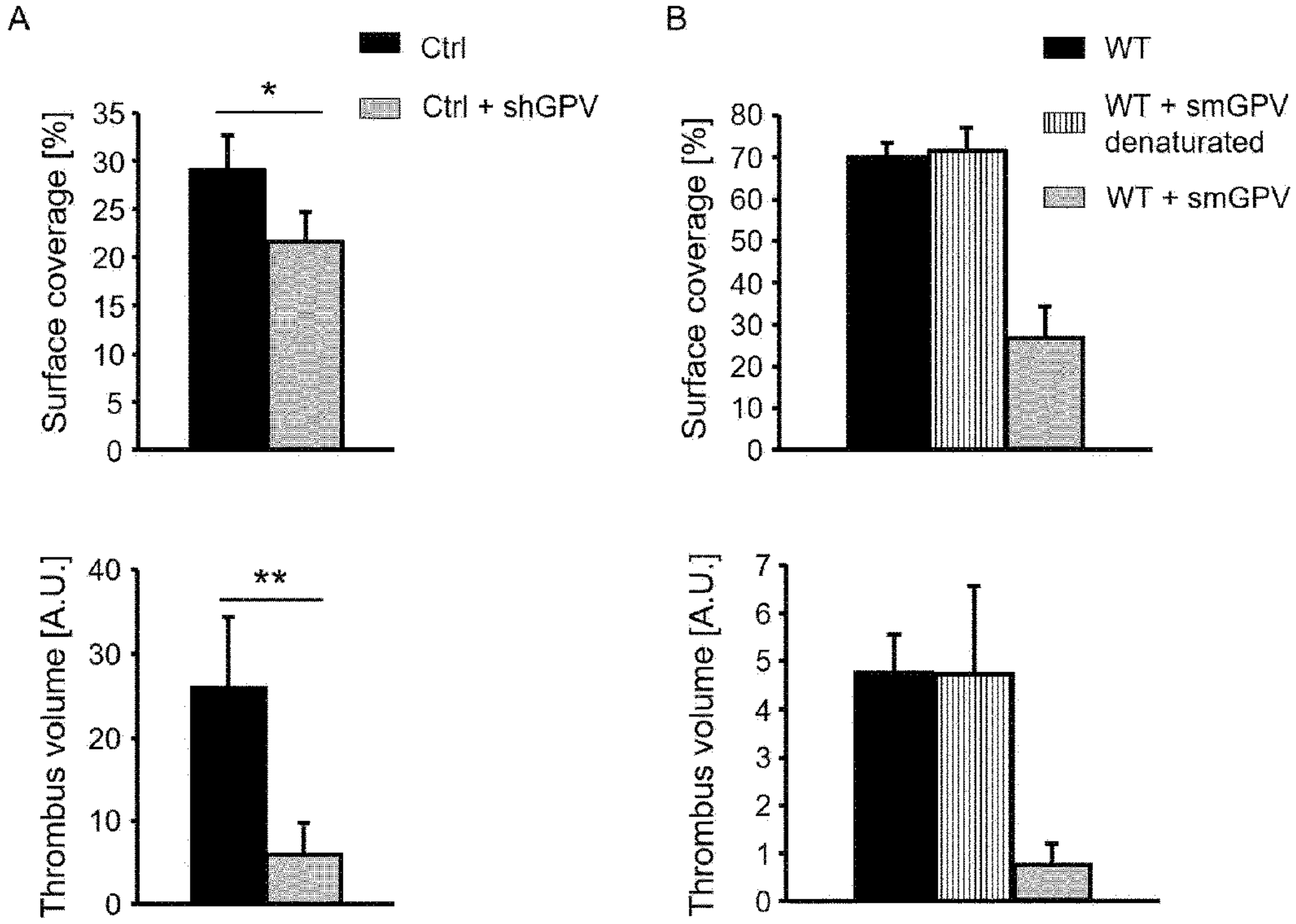


Figure 4


A
B
Ctrl
Ctrl + shGPV
WT
WT + smGPV denaturated
WT + smGPV
Surface coverage [%]
Thrombus volume [A.U.]
*
**
0
5
10
15
20
25
30
35
40
50
60
70
80
1
2
3
4
6
7