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(73) Patenthaver: **CSL Behring GmbH, Emil-von-Behring-Strasse 76, 35041 Marburg, Tyskland**

(72) Opfinder: **Weimer, Thomas, Richard-Wagner-Strasse 8, 35075 Gladenbach, Tyskland**
Schulte, Stefan, Bauerbacher Strasse 46, 35043 Marburg, Tyskland
Metzner, Hubert, Im Boden 6, 35041 Marburg, Tyskland
Lang, Wiegand, Akazienweg 2, 35091 Cölbe, Tyskland
Kronthaler, Ulrich, Stefanienstraße 5, 82041 Deisenhofen, Tyskland
Lind, Holger, Elisabeth-von-Thadden-Straße 8, 35037 Marburg, Tyskland

(74) Fuldmægtig i Danmark: **Patrade A/S, Fredens Torv 3A, 8000 Århus C, Danmark**

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DESCRIPTION

Field of the invention:

[0001] The present invention relates to modified nucleic acid sequences coding for coagulation factor VIII (FVIII) and for non-modified von Willebrand factor (VWF) as well as complexes thereof and their derivatives, recombinant expression vectors containing such nucleic acid sequences, host cells transformed with such recombinant expression vectors, recombinant polypeptides and derivatives coded for by said nucleic acid sequences which recombinant polypeptides and derivatives do have biological activities together with prolonged in vivo half-life and/or improved in vivo recovery compared to the unmodified wild-type protein. The invention also relates to corresponding FVIII sequences that result in improved expression yield. The present invention further relates to processes for the manufacture of such recombinant proteins and their derivatives. The invention also relates to a transfer vector for use in human gene therapy, which comprises such modified nucleic acid sequences.

Background of the invention:

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

[0003] In plasma FVIII exists mostly as a noncovalent complex with VWF and its coagulant function is to accelerate factor IXa dependent conversion of factor X to Xa Due to the complex formation of FVIII and VWF it was assumed for a long time that FVIII and VWF functions are two functions of the same molecule. Only in the seventies it became clear that FVIII and VWF are separate molecules that form a complex under physiologic conditions. In the eighties then the dissociation constant of about 0.2 nmol/L was determined (Leyte et al., Biochem J 1989, 257: 679-683) and the DNA sequence of both molecules was studied.

[0004] Classic hemophilia or hemophilia A is an inherited bleeding disorder. It results from a chromosome X-linked deficiency of blood coagulation FVIII, and affects almost exclusively males with an incidence of between one and two individuals per 10.000. The X-chromosome defect is transmitted by female carriers who are not themselves hemophiliacs. The clinical manifestation of hemophilia A is an increased bleeding tendency. Before treatment with FVIII concentrates was introduced the mean life span for a person with severe hemophilia was less than 20 years. The use of concentrates of FVIII from plasma has considerably improved the situation for the hemophilia A patients increasing the mean life span extensively, giving most of them the possibility to live a more or less normal life. However, there have been certain problems with the plasma derived concentrates and their use, the most serious of which have

been the transmission of viruses. So far, viruses causing hepatitis B, non-A non-B hepatitis and AIDS have hit the population seriously. Since then different virus inactivation methods and new highly purified FVIII concentrates have recently been developed which established a very high safety standard also for plasma derived FVIII.

[0005] The cloning of the cDNA for FVIII (Wood et al. 1984. Nature 312:330-336; Vehar et al. 1984. Nature 312:337-342) made it possible to express FVIII recombinantly leading to the development of several recombinant FVIII products, which were approved by the regulatory authorities between 1992 and 2003. The fact that the central B domain of the FVIII polypeptide chain residing between amino acids Arg-740 and Glu-1649 does not seem to be necessary for full biological activity has also led to the development of a B domain deleted FVIII.

[0006] The mature FVIII molecule consists of 2332 amino acids which can be grouped into three homologous A domains, two homologous C domains and a B Domain which are arranged in the order: A1-A2-B-A3-C1-C2. The complete amino acid sequence of mature human FVIII is shown in SEQ ID NO:15. During its secretion into plasma FVIII is processed intracellularly into a series of metal-ion linked heterodimers as single chain FVIII is cleaved at the B-A3 boundary and at different sites within the B-domain. This processing leads to heterogeneous heavy chain molecules consisting of the A1, the A2 and various parts of the B-domain which have a molecular size ranging from 90 kDa to 200 kDa. The heavy chains are bound via a metal ion to the light chains, which consist of the A3, the C1 and the C2 domain (Saenko et al. 2002. Vox Sang. 83:89-96). In plasma this heterodimeric FVIII binds with high affinity to von Willebrand Factor (VWF), which protects it from premature catabolism. The half-life of non-activated FVIII bound to VWF is about 12 hours in plasma.

[0007] Coagulation FVIII is activated via proteolytic cleavage by FXa and thrombin at amino acids Arg372 and Arg740 within the heavy chain and at Arg1689 in the light chain resulting in the release of von Willebrand Factor and generating the activated FVIII heterotrimer which will form the tenase complex on phospholipid surfaces with FIXa and FX provided that Ca^{2+} is present. The heterotrimer consists of the A1 domain, a 50 kDa fragment, the A2 domain, a 43 kDa fragment and the light chain (A3-C1-C2), a 73 kDa fragment. Thus the active form of FVIII (FVIIIa) consists of an A1-subunit associated through the divalent metal ion linkage to a thrombin-cleaved A3-C1-C2 light chain and a free A2 subunit relatively loosely associated with the A1 and the A3 domain.

[0008] To avoid excessive coagulation, FVIIIa must be inactivated soon after activation. The inactivation of FVIIIa via activated Protein C (APC) by cleavage at Arg336 and Arg562 is not considered to be the major rate-limiting step. It is rather the dissociation of the non covalently attached A2 subunit from the heterotrimer which is thought to be the rate limiting step in FVIIIa inactivation after thrombin activation (Fay et al. 1991. J. Biol. Chem. 266 8957, Fay & Smudzin 1992. J. Biol. Chem. 267:13246-50). This is a rapid process, which explains the short half-life of FVIIIa in plasma, which is only 2.1 minutes (Saenko et al. 2002. Vox Sang. 83:89-96).

[0009] In severe hemophilia A patients undergoing prophylactic treatment FVIII has to be

administered intravenously (i.v.) about 3 times per week due to the short plasma half-life of FVIII of about 12 to 14 hours. Each i.v. administration is cumbersome, associated with pain and entails the risk of an infection especially as this is mostly done at home by the patients themselves or by the parents of children being diagnosed for hemophilia A.

[0010] It would thus be highly desirable to create a FVIII with increased functional half-life allowing the manufacturing of pharmaceutical compositions containing FVIII, which have to be administered less frequently.

[0011] Several attempts have been made to prolong the half-life of non-activated FVIII either by reducing its interaction with cellular receptors (WO 03/093313A2, WO 02/060951A2), by covalently attaching polymers to FVIII (WO 94/15625, WO 97/11957 and US 4970300), by encapsulation of FVIII (WO 99/55306), by introduction of novel metal binding sites (WO 97/03193), by covalently attaching the A2 domain to the A3 domain either by peptidic (WO 97/40145 and WO 03/087355) or disulfide linkage (WO 02/103024A2) or by covalently attaching the A1 domain to the A2 domain (WO2006/108590).

[0012] Another approach to enhance the functional half-life of FVIII or VWF is by PEGylation of FVIII (WO 2007/126808, WO 2006/053299, WO 2004/075923) or by PEGylation of VWF (WO 2006/071801) which pegylated VWF by having an increased half-life would indirectly also enhance the half-life of FVIII present in plasma.

[0013] As none of the above described approaches has yet resulted in an approved FVIII pharmaceutical and as introducing mutations into the FVIII wild-type sequence or introducing chemical modifications entails at least a theoretical risk of creating immunogenic FVIII variants there is an ongoing need to develop modified coagulation factor VIII molecules which exhibit prolonged half-life.

[0014] In view of a potential thrombogenic risk it is more desirable to prolong the half-life of the non-activated form of FVIII than that of FVIIIa.

[0015] VWF, which is missing, functionally defect or only available in reduced quantity in different forms of von Willebrand disease (VWD), is a multimeric adhesive glycoprotein present in the plasma of mammals, which has multiple physiological functions. During primary hemostasis VWF acts as a mediator between specific receptors on the platelet surface and components of the extracellular matrix such as collagen. Moreover, VWF serves as a carrier and stabilizing protein for procoagulant FVIII. VWF is synthesized in endothelial cells and megakaryocytes as a 2813 amino acid precursor molecule. The amino acid sequence and the cDNA sequence of wild-type VWF are disclosed in Collins et al. 1987, Proc Natl. Acad. Sci. USA 84:4393-4397. The precursor polypeptide, pre-pro-VWF, consists of a 22-residue signal peptide, a 741- residue pro-peptide and the 2050-residue polypeptide found in mature plasma VWF (Fischer et al., FEBS Lett. 351: 345-348, 1994). After cleavage of the signal peptide in the endoplasmatic reticulum a C-terminal disulfide bridge is formed between two monomers of VWF. During further transport through the secretory pathway 12 N-linked and 10 O-linked

carbohydrate side chains are added. More important, VWF dimers are multimerized via N-terminal disulfide bridges and the propeptide of 741 amino acids length is cleaved off by the enzyme PACE/furin in the late Golgi apparatus. The propeptide as well as the high-molecular-weight multimers of VWF (VWF-HMWM) are stored in the Weibel-Pallade bodies of endothelial cells or in the α-Granules of platelets.

[0016] Once secreted into plasma the protease ADAMTS13 cleaves VWF within the A1 domain of VWF. Plasma VWF therefore consists of a whole range of multimers ranging from single dimers of 500 kDa to multimers consisting of up to more than 20 dimers of a molecular weight of over 10,000 kDa. The VWF-HMWM hereby having the strongest hemostatic activity, which can be measured in ristocetin cofactor activity (VWF:RCo). The higher the ratio of VWF:RCo/VWF antigen, the higher the relative amount of high molecular weight multimers.

[0017] Defects in VWF are causal to von Willebrand disease (VWD), which is characterized by a more or less pronounced bleeding phenotype. VWD type 3 is the most severe form in which VWF is completely missing, VWD type 1 relates to a quantitative loss of VWF and its phenotype can be very mild. VWD type 2 relates to qualitative defects of VWF and can be as severe as VWD type 3. VWD type 2 has many sub forms some of them being associated with the loss or the decrease of high molecular weight multimers. Von VWD type 2a is characterized by a loss of both intermediate and large multimers. VWD type 2B is characterized by a loss of highest-molecular-weight multimers.

[0018] VWD is the most frequent inherited bleeding disorder in humans and can be treated by replacement therapy with concentrates containing VWF of plasmatic or recombinant origin. VWF can be prepared from human plasma as for example described in EP 05503991. EP 0784632 describes a method for isolating recombinant VWF.

[0019] In plasma FVIII binds with high affinity to VWF, which protects it from premature catabolism and thus, plays in addition to its role in primary hemostasis a crucial role to regulate plasma levels of FVIII and as a consequence is also a central factor to control secondary hemostasis. The half-life of non-activated FVIII bound to VWF is about 12 to 14 hours in plasma. In von Willebrand disease type 3, where no or almost no VWF is present, the half-life of FVIII is only about 6 hours, leading to symptoms of mild to moderate hemophilia A in such patients due to decreased concentrations of FVIII. The stabilizing effect of VWF on FVIII has also been used to aid recombinant expression of FVIII in CHO cells (Kaufman et al. 1989, Mol Cell Biol).

[0020] Until today the standard treatment of Hemophilia A and VWD involves frequent intravenous infusions of preparations of FVIII and VWF concentrates or of concentrates comprising a complex of FVIII and VWF derived from the plasmas of human donors or in case of FVIII that of pharmaceutical preparations based on recombinant FVIII. While these replacement therapies are generally effective, e.g. in severe hemophilia A patients undergoing prophylactic treatment FVIII has to be administered intravenously (i.v.) about 3 times per week due to the short plasma half life of FVIII of about 12 hours. Already above levels of 1% of the

FVIII activity in non-hemophiliacs, e.g. by a raise of FVIII levels by 0.01 U/ml, severe hemophilia A is turned into moderate hemophilia A. In prophylactic therapy dosing regimes are designed such that the trough levels of FVIII activity do not fall below levels of 2-3% of the FVIII activity in non-hemophiliacs. Each i.v. administration is cumbersome, associated with pain and entails the risk of an infection especially as this is mostly done in home treatment by the patients themselves or by the parents of children being diagnosed for hemophilia A. In addition the frequent i.v. injections inevitably result in scar formation, interfering with future infusions. As prophylactic treatment in severe hemophilia is started early in life, with children often being less than 2 years old, it is even more difficult to inject FVIII 3 times per week into the veins of such small patients. For a limited period, implantation of port systems may offer an alternative. Despite the fact that repeated infections may occur and ports can cause inconvenience during physical exercise, they are nevertheless typically considered as favorable as compared to intravenous injections.

[0021] In the prior art fusions of coagulation factors to albumin (WO 01/79271), alpha-fetoprotein (WO 2005/024044) and immunoglobulin (WO 2004/101740) as half-life enhancing polypeptides have been described. These were taught to be attached to the carboxy- or the amino-terminus or to both termini of the respective therapeutic protein moiety, occasionally linked by peptidic linkers, preferably by linkers consisting of glycine and serine.

[0022] Ballance et al. (WO 01/79271) described N- or C-terminal fusion polypeptides of a multitude of different therapeutic polypeptides fused to human serum albumin. Long lists of potential fusion partners are described without disclosing experimental data for almost any of these polypeptides whether or not the respective albumin fusion proteins actually retain biological activity and have improved properties. Among said list of therapeutic polypeptides also FVIII and VWF are mentioned.

[0023] A C-terminal fusion would not have been seriously considered by the man skilled in the art as the C2 domain of FVIII at the very C-terminal part of FVIII between amino acid 2303 and 2332 of FVIII comprises a platelet membrane binding site which is essential for FVIII function. This is why there are many amino acid mutations known in this region which lead to hemophilia A. It was thus surprising that a relatively large heterologous polypeptide like albumin can be fused to the C-terminal part of FVIII without preventing FVIII function by preventing platelet binding. In addition, the C2 domain also contains a binding site for VWF. This site together with the amino acid sequence 1649-1689 is responsible for the high affinity binding of FVIII to VWF. Therefore, a man skilled in the art would also not have expected that a FVIII with a C-terminal albumin fusion would retain its binding to VWF.

[0024] It was surprisingly found that in contrast to the prediction by Ballance et al. an albumin fusion to the N-terminus of FVIII was not secreted into the culture medium. Therefore and because of the reasons detailed above it was now even more surprisingly found that a FVIII fused at its C-terminal part to albumin is secreted into the culture medium and retains its biological function including binding to membranes of activated platelets and to VWF.

[0025] It was also surprising to find that the modified FVIII of the invention shows an increase of in vivo recovery by about 20% compared to the wild type FVIII.

Description of the invention

[0026] It is an objective of this invention to provide a modified FVIII or complexes of modified FVIII with non-modified VWF, with enhanced in vivo half-life.

[0027] The term "modified FVIII" in the sense of the invention means FVIII polypeptides which are fused to half-life enhancing polypeptides, encompassing also natural alleles, variants, deletions and insertions of FVIII.

[0028] It is another objective of this invention to provide a modified FVIII as well as complexes of modified FVIII with non-modified VWF with improved in vivo recovery.

[0029] Another objective of the invention is that this modified FVIII or complexes of modified FVIII with non-modified VWF can be expressed by mammalian cells and retain their respective biological activities.

[0030] In summary, surprisingly the modified FVIII or complexes of modified FVIII with non-modified VWF of the invention have retained biological activity, increased in vivo half-life and in vivo recovery.

[0031] An additional potential benefit of those embodiments of the present invention in which the FVIII is modified and in which the A2 domain remains only non covalently attached to the A3 domain after activation is that only the half-life of the non-activated form of FVIII is increased, whereas the half-life of the activated form of FVIII remains essentially the same, which might result in a decreased risk of thrombogenicity as compared to FVIII variants which lead to a stabilization of the activated form of FVIII.

[0032] The modified FVIII or complexes of modified FVIII with non-modified VWF of the invention can be generated by fusing a half-life enhancing protein (HLEP) moiety to the C-terminal part of FVIII.

[0033] HLEPs in the sense of the present invention are selected from a group consisting of members of human albumin.

[0034] The present invention therefore relates to a modified FVIII or of modified FVIII with non-modified VWF having at the C-terminal part of the modified FVIII a fusion to a HLEP, characterized in that the modified FVIII or the complex of modified FVIII with non-modified VWF has prolonged functional half-life compared to the functional half-life of the wild-type FVIII or the complex of wild-type VWF and wild-type FVIII.

[0035] The present invention also relates to C-terminal fusions to more than one HLEP wherein the HLEP, which is fused several times.

[0036] The present invention also relates to a modified FVIII having at the C-terminal part a fusion to a HLEP, characterized in that the modified FVIII or the complex of modified FVIII with non-modified VWF has improved in vivo recovery compared to the in vivo recovery of the wild-type FVIII or the complex of wild-type VWF and wild-type FVIII.

[0037] Another embodiment of the invention are modified FVIII polypeptides having at the C-terminal part a fusion to a HLEP, characterized in that the modified FVIII is secreted into a fermentation medium at a higher yield as a wild-type FVIII.

[0038] Another aspect of the invention are polynucleotides or combinations of polynucleotides encoding the modified FVIII.

[0039] The invention further relates to plasmids or vectors comprising a polynucleotide described herein, to host cells comprising a polynucleotide or a plasmid or vector described herein.

[0040] Another aspect of the invention is a method of producing a modified FVIII or a complex of modified FVIII with non-modified VWF, comprising:

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

[0041] The invention further pertains to pharmaceutical compositions comprising a modified FVIII or a complex of modified FVIII with non-modified VWF, a polynucleotide, or a plasmid or vector described herein.
Yet another aspect of the invention is the use of a modified FVIII or a complex of modified FVIII with non-modified VWF, one or more polynucleotides, or one or more plasmids or vectors, or of host cells according to this invention for the manufacture of a medicament for the treatment or prevention of a blood coagulation disorder.

Detailed description of the invention

[0042] The invention pertains to a complex comprising FVIII and VWF or one of its individual polypeptidic components wherein the FVIII of said complex is fused at the C-terminal part of its primary translation product to the N-terminal part of a half-life enhancing polypeptide (HLEP).

[0043] The invention also pertains to a modified FVIII or a complex comprising modified FVIII and non-modified wherein the modified FVIII is fused at a C-terminal part of the primary translation polypeptide of FVIII to the N-terminal part of a HLEP.

[0044] In preferred embodiments the invention pertains to a modified FVIII or a complex comprising modified FVIII and non-modified, wherein

1. (a) the modified FVIII has a prolonged functional half-life compared to the functional half-life of wild-type FVIII or
2. (b) the complex comprising modified FVIII and non-modified VWF has a prolonged functional half-life compared to the functional half-life of the corresponding complex comprising wild-type FVIII and wild-type VWF.

[0045] A preferred embodiment of the invention is a modified polypeptide or a complex comprising said modified polypeptide or a complex comprising said modified polypeptides as described above, wherein the modified polypeptide has a functional half-life increased by at least 25% as compared to the functional half-life of the corresponding wild-type polypeptide or the complex comprising said modified polypeptide or a complex comprising said modified polypeptides has a functional half-life increased by at least 25% as compared to the corresponding complex of wild-type FVIII and wild-type VWF.

[0046] Another embodiment of the invention is a modified FVIII or a complex comprising modified FVIII and non-modified VWF, wherein

1. (a) the modified FVIII has a prolonged antigen half-life compared to the antigen half-life of wild-type FVIII or
2. (b) the complex comprising modified FVIII and non-modified VWF has a prolonged antigen half-life compared to the antigen half-life of the corresponding complex comprising wild-type FVIII and wild-type VWF.

[0047] A preferred embodiment of the invention is a modified polypeptide or a complex comprising said modified polypeptide or a complex comprising said modified polypeptides as described above, wherein the modified polypeptide has an antigen half-life increased by at least 25% as compared to the antigen half-life of the corresponding wild-type polypeptide or the complex comprising said modified polypeptide or a complex comprising said modified polypeptides has an antigen half-life increased by at least 25% as compared to the corresponding complex of wild-type FVIII and wild-type VWF.

[0048] Still another embodiment of the invention is a modified FVIII or a complex comprising modified FVIII and non-modified VWF , wherein

1. (a) the modified FVIII has an increased in vivo recovery compared to the in vivo recovery

of wild-type FVIII or

2. (b) the complex comprising modified FVIII and non-modified VWF has an increased in vivo recovery compared to the in vivo recovery of the corresponding complex comprising wild-type FVIII and wild-type VWF .

[0049] Another preferred embodiment of the invention is a modified polypeptide or a complex comprising said modified polypeptide or a complex comprising said modified polypeptides as described above, wherein the modified polypeptide has an in vivo recovery increased by at least 10% as compared to the in vivo recovery of the corresponding wild-type polypeptide or the complex comprising said modified polypeptide or a complex comprising said modified polypeptides has an in vivo recovery increased by at least 10% as compared to the corresponding complex of wild-type FVIII and wild-type VWF.
Another preferred embodiment of the invention is

1. (a) a modified polypeptide or a complex comprising said modified polypeptide or a complex comprising said modified polypeptides as described above, wherein at least one polypeptidic component of said complex is fused at the C-terminal amino acid of its primary translation product to the N-terminal part of a HLEP or
2. (b) a modified polypeptide or a complex comprising said modified polypeptide or a complex comprising said modified polypeptides as described above, wherein at least one polypeptidic component of said complex is fused at the C-terminal part of its primary translation product to the N-terminal amino acid of a HLEP or
3. (c) a modified polypeptide or a complex comprising said modified polypeptide or a complex comprising said modified polypeptides as described above, wherein at least one polypeptidic component of said complex is fused at the C-terminal amino acid of its primary translation product to the N-terminal amino acid of a HLEP.

[0050] Another preferred embodiment of the invention is a modified polypeptide or a complex comprising said modified polypeptide or a complex comprising said modified polypeptides as described above, wherein the modified polypeptide has at least 10% of the biological activity of wild-type polypeptide or the complex comprising the modified polypeptide or a complex comprising said modified polypeptides has at least 10% of the biological activity of the corresponding complex of wild-type FVIII and wild-type VWF.

[0051] Also comprised in the present invention is a method of preparing a modified having increased functional half-life, comprising fusing the N-terminal part of a half-life-enhancing polypeptide to a C-terminal part of the primary translation polypeptide of the FVIII as well as a method of preparing a complex comprising modified FVIII and non-modified VWF by mixing a modified FVIII prepared by the method described above with wild-type VWF prepared by the method described above.

[0052] Also encompassed in the invention is the use of a modified FVIII as prepared by the method described above and wild-type VWF for the manufacture of a combined pharmaceutical preparation for simultaneous, separate or sequential use in the therapy of bleeding disorders, preferentially in the therapy of hemophilia A and/or von Willebrand disease.

[0053] The "functional half-life" according to the present invention is the half-life of the biological activity of the modified FVIII or a complex of modified FVIII with non-modified VWF once it has been administered to a mammal and can be measured in vitro in blood samples taken at different time intervals from said mammal after the modified FVIII or the complex of modified FVIII with non-modified VWF has been administered.

[0054] The phrases "fusing" or "fused" refer to the addition of amino acids to the C-terminal part of FVIII. When referring herein to a "fusion to the C-terminal amino acid of FVIII" this means a fusion exactly to the C-terminal amino acid of FVIII at amino acid 2332 of the mature wild-type FVIII cDNA sequence. Mature FVIII meaning the respective polypeptide after cleavage of the propeptide. However the invention also encompasses a "fusion to the C-terminal part of FVIII" in the sense of this invention may also include a fusion to a FVIII molecule respectively in which one or more amino acid position up to n amino acids from the C-terminal amino acid of FVIII are deleted. The figure n is an integer that should not be greater than 5%, preferably not greater than 1% of the total number of amino acids of the FVIII. Usually, n is 20, preferably 15, more preferably 10, still more preferably 5 or less (e.g. 1, 2, 3, 4 or 5).

[0055] In one embodiment, the modified FVIII has the following structure:

N - FVIII - C -L1- H, [formula 1]

wherein

N is an N-terminal part of FVIII,

L1 is a chemical bond or a linker sequence

H is a HLEP, and

C is a C-terminal part of FVIII

[0056] L1 may be a chemical bond or a linker sequence consisting of one or more amino acids, e.g. of 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 coagulation factor. Examples of suitable amino acids present in L1 include Gly and Ser.

[0057] Preferred HLEP sequences are described infra. 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.

[0058] The modified FVIII or the complex of the modified FVIII with the non-modified VWF, of the invention 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 FVIII in tandem, e.g. as successive repeats.

[0059] FVIII may be processed proteolytically at various stages. For example, as mentioned supra, during its secretion into plasma single chain FVIII is cleaved intracellularly at the B-A3 boundary and at different sites within the B-domain. The heavy chain is bound via a metal ion to the light chain having the domain structure A3-C1-C2. FVIII is activated via proteolytic cleavage at amino acids Arg372 and Arg740 within the heavy chain and at Arg1689 in the light chain generating the activated FVIII heterotrimer consisting of the A1 domain, the A2 domain, and the light chain (A3-C1-C2), a 73 kDa fragment. Thus the active form of FVIII (FVIIIa) consists of an A1-subunit associated through the divalent metal ion linkage to a thrombin-cleaved A3-C1-C2 light chain and a free A2 subunit relatively loosely associated with the A1 and the A3 domain.

[0060] Accordingly, the present invention encompasses also modified FVIII that is not present as a single chain polypeptide but consists of several polypeptides (e.g. one or two or three) that are associated with each other via non-covalent linkages.

[0061] Preferably N - FVIII - C comprises the full length sequence of FVIII. Also encompassed are N-terminal, C-terminal or internal deletions of FVIII as long as the biological activity of FVIII is retained. The biological activity is retained in the sense of the invention if the FVIII with deletions retains at least 10%, preferably at least 25%, more preferably at least 50%, most preferably at least 75% of the biological activity of wild-type FVIII. The biological activity of FVIII can be determined by the artisan as described below.

[0062] A suitable test to determine the biological activity of FVIII is for example the one stage or the two stage coagulation assay (Rizza et al. 1982. Coagulation assay of FVIII:C and FIXa in Bloom ed. The Hemophilias. NY Churchchill Livingston 1992) or the chromogenic substrate FVIII:C assay (S. Rosen, 1984. Scand J Haematol 33: 139-145, suppl.). The content of these references is incorporated herein by reference.

[0063] The cDNA sequence and the amino acid sequence of the mature wild-type form of human blood coagulation FVIII are shown in SEQ ID NO:14 and SEQ ID NO:15, respectively. The reference to an amino acid position of a specific sequence means the position of said amino acid in the FVIII wild-type protein and does not exclude the presence of mutations, e.g. deletions, insertions and/or substitutions at other positions in the sequence referred to. For example, a mutation in "Glu2004" referring to SEQ ID NO:15 does not exclude that in the modified homologue one or more amino acids at positions 1 through 2332 of SEQ ID NO:15

are missing.

[0064] The terms "blood coagulation Factor VIII", "Factor VIII" and "FVIII" are used interchangeably herein. "Blood coagulation Factor VIII" includes wild-type blood coagulation FVIII as well as derivatives of wild-type blood coagulation FVIII having the procoagulant activity of wild-type blood coagulation FVIII. Derivatives may have deletions, insertions and/or additions compared with the amino acid sequence of wild-type FVIII. The term FVIII includes proteolytically processed forms of FVIII, e.g. the form before activation, comprising heavy chain and light chain.

[0065] The term "FVIII" includes any FVIII variants or mutants having at least 25%, more preferably at least 50%, most preferably at least 75% of the biological activity of wild-type factor VIII.

[0066] As non-limiting examples, FVIII molecules include FVIII mutants preventing or reducing APC cleavage (Amano 1998. Thromb. Haemost. 79:557-563), FVIII mutants further stabilizing the A2 domain (WO 97/40145), FVIII mutants resulting in increased expression (Swaroop et al. 1997. JBC 272:24121-24124), FVIII mutants reducing its immunogenicity (Lollar 1999. Thromb. Haemost. 82:505-508), FVIII reconstituted from differently expressed heavy and light chains (Oh et al. 1999. Exp. Mol. Med. 31:95-100), FVIII mutants reducing binding to receptors leading to catabolism of FVIII like HSPG (heparan sulfate proteoglycans) and/or LRP (low density lipoprotein receptor related protein) (Ananyeva et al. 2001. TCM, 11:251-257), disulfide bond-stabilized FVIII variants (Gale et al., 2006. J. Thromb. Hemost. 4:1315-1322), FVIII mutants with improved secretion properties (Miao et al., 2004. Blood 103:3412-3419), FVIII mutants with increased cofactor specific activity (Wakabayashi et al., 2005. Biochemistry 44:10298-304), FVIII mutants with improved biosynthesis and secretion, reduced ER chaperone interaction, improved ER-Golgi transport, increased activation or resistance to inactivation and improved half-life (summarized by Pipe 2004. Sem. Thromb. Hemost. 30:227-237). All of these FVIII mutants and variants are incorporated herein by reference in their entirety.

[0067] VWF may be processed proteolytically at various stages. For example, as mentioned supra, the protease ADAMTS13 cleaves VWF within the A2 domain of VWF. Accordingly, the present invention encompasses also modified VWF which has been cleaved proteolytically e.g. by ADAMTS13. Such cleavage would result in multimeric chains of VWF which comprise at their ends at least one or at most two monomers of VWF which have been cleaved by ADAMTS 13.

[0068] Preferably N - VWF - C comprises the full length sequence of VWF. Also encompassed are N-terminal, C-terminal or internal deletions of VWF as long as the biological activity of VWF is retained. The biological activity is retained in the sense of the invention if the VWF with deletions retains at least 10%, preferably at least 25%, more preferably at least 50%, most preferably at least 75% of the biological activity of wild-type VWF. The biological activity of wild-type VWF can be determined by the artisan using methods for ristocetin cofactor activity

(Federici AB et al. 2004. Haematologica 89:77-85), binding of VWF to GP Iba of the platelet glycoprotein complex Ib-V-IX (Sucker et al. 2006. Clin Appl Thromb Hemost. 12:305-310), or a collagen binding assay (Kallas & Talpsep. 2001. Annals of Hematology 80:466-471).

[0069] "FVIII" and/or "VWF" within the above definition also include natural allelic variations that may exist and occur from one individual to another. "FVIII" and/or "VWF" within the above definition further includes variants of FVIII and or VWF. Such variants differ in one or more amino acid residues from the wild-type sequence. Examples of such differences may include as conservative amino acid substitutions, i.e. substitutions within groups of amino acids with similar characteristics, e.g. (1) small amino acids, (2) acidic amino acids, (3) polar amino acids, (4) basic amino acids, (5) hydrophobic amino acids, and (6) aromatic amino acids. Examples of such conservative substitutions are shown in the following table.

Table 1:

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

[0070] One or more HLEPs may be fused to the C-terminal part of FVIII preferably as not to interfere with the binding capabilities of FVIII for example to VWF, platelets or FIX.

[0071] Once FVIII is endogenously activated during coagulation in vivo, it may be no longer desirable to maintain the increased functional half-life of the now activated FVIII as this might lead to thrombotic complications what is already the case for a wild-type activated coagulation factor as FVIIa (Aledort 2004. J Thromb Haemost 2:1700-1708) and what may be more relevant if the activated factor would have an increased functional half-life. It is therefore another objective of the present invention to provide long-lived FVIII molecules, which after endogenous activation in vivo or after availability of a cofactor do have a functional half-life comparable to that of unmodified FVIII. This can by way of non-limiting example be achieved by introducing a cleavage site for example for a coagulation factor between the C-terminal part of FVIII and the HLEP. With such FVIII-HLEP connecting sequences the activation of the FVIII chimeric protein of the invention will lead to a concomitant complete separation of FVIIIa from the HLEP moiety. Accordingly, in one embodiment, the functional half-life of the endogenously activated modified FVIII is substantially the same as that of the activated wild-type FVIII (e.g. ±15%, preferably ±10%).

[0072] In yet another embodiment of the invention, however, one or more of the proteolytical cleavage sites, preferably the thrombin cleavage sites at Arg740 and/or Arg372, are mutated or deleted in order to prevent cleavage and result in an insertion protein which displays improved properties like enhanced functional half-life even as an activated molecule.

[0073] In another embodiment of the invention the FVIII proteins of the invention may be expressed as two separate chains (see infra).

[0074] The modified FVIII according to this invention may be a single chain polypeptide, or it may be composed of two or three polypeptide chains that are associated via non-covalent linkages, due to proteolytic processing.

[0075] In another embodiment of the invention, the amino acids at or near the PACE/Furin cleavage site (Arg1648) are mutated or deleted in order to prevent cleavage by PACE/Furin. This is thought to result in a one-chain FVIII/HLEP fusion molecule with improved half-life.

[0076] In one embodiment of the invention, the modified FVIII of the invention exhibits an increased functional half-life compared to the corresponding FVIII form containing no integrated HLEP and/or to the wild-type form FVIII. The functional half-life e.g. can be determined in vivo in animal models of hemophilia A, like FVIII knockout mice, in which one would expect a longer lasting hemostatic effect as compared to wild-type FVIII. The hemostatic effect could be tested for example by determining time to arrest of bleeding after a tail clip.

[0077] The functional half-life in one embodiment of the invention is the half-life of the biological activity of the FVIII once it has been administered to a mammal and is measured in vitro. The functional half-life of the modified FVIII according to the invention is greater than that of the FVIII lacking the modification as tested in the same species. The functional half-life is preferably increased by at least 10%, preferably 25%, more preferably by at least 50%, and even more preferably by at least 100% compared to the wild-type form of FVIII.

[0078] The functional half-life of a modified FVIII comprising a HLEP modification, can be determined by administering the respective modified FVIII (and in comparison wild-type FVIII) to rats, rabbits or other experimental animal species intravenously or subcutaneously and following the elimination of the biological activity of said modified or respectively non-modified coagulation factor in blood samples drawn at appropriate intervals after application. Suitable test methods are the activity tests described herein.

[0079] As a surrogate marker for the half-life of biological activity also the levels of antigen of the modified or respectively wild-type FVIII or the levels of wild-type VWF can be measured. Thus also encompassed by the invention are modified FVIII molecules having at the C-terminal part of FVIII a fusion to a HLEP, characterized in that the modified FVIII o or the complex of modified FVIII with non-modified VWF has a prolonged half-life of the FVIII antigen compared to the half-life of the FVIII antigen lacking said insertion. The "half-life of the FVIII antigen" according to the present invention is the half-life of the antigen of the FVIII once it has been administered to a mammal and is measured in vitro. Antigen test methods based on specific antibodies in an enzyme immunoassay format as known to the artisan and commercially available (e.g. Dade Behring, Instrumentation Laboratory, Abbott Laboratories, Diagnostica Stago). Functional and antigen half-lives can be calculated using the time points of the beta

phase of elimination according to the formula $t_{1/2}$ = ln2 / k, whereas k is the slope of the regression line.

[0080] In another embodiment, the functional half-life of the endogenously activated modified FVIII is prolonged compared to that of the activated wild-type FVIII. The increase may be more than 15%, for example at least 20% or at least 50%. Again, such functional half-life values can be measured and calculated as described for functional half-lives supra. Increased half-lives of the endogenously activated modified FVIII molecules may be beneficial in situations were only very low levels of FVIII are available that therefore are not thrombogenic. Such situations may occur e.g. upon gene therapy treatment where often only low expression rates can be achieved. Therefore, such stabilized FVIII molecules might be beneficial in e.g. gene therapy despite a thrombogenic risk connected to such FVIII molecules if administered as proteins in high or physiologic doses.

[0081] In another embodiment of the invention, the modified FVIII of the invention exhibits an improved in vivo recovery compared to the wild-type FVIII . The in vivo recovery can be determined in vivo for example in normal animals or in animal models of hemophilia A, like FVIII knockout mice, in which one would expect an increased percentage of the modified FVIII of the invention be found by antigen or activity assays in the circulation shortly (5 to 10 min.) after i.v. administration compared to the corresponding wild-type FVIII or wild-type VWF.

[0082] The in vivo recovery is preferably increased by at least 10%, more preferably by at least 20%, and even more preferably by at least 40% compared to wild-type form FVIII or to wild-type VWF.

[0083] In yet another embodiment of the invention immunoglobulin constant regions or portions thereof are used as HLEPs. Preferably the Fc region comprised of a CH2 and CH3 domain and a hinge region of an IgG, more preferably of an IgG1 or fragments or variants thereof are used, variants including mutations which enhance binding to the neonatal Fc receptor (FcRn).

[0084] It is another objective of the present invention to provide long-lived FVIII molecules, which after proteolytic processing in vivo do have a functional half-life comparable to that of an unmodified FVIII. This can be achieved by maintaining certain cleavage sites in the modified FVIII leading to a proteolytic cleavage for example when in contact with activated coagulation factors, which separates the FVIII from the HLEP. Accordingly, in one embodiment, the functional half-life of the proteolytically processed modified FVIII is substantially the same as that of the non-modified VWF lacking the modification, and/or it is substantially the same as that of the wild-type VWF (e.g. ±15%, preferably ±10%).

[0085] Still another embodiment of the invention are modified FVIII polypeptides which are fused to a HLEP for example albumin at the C-terminal part of the FVIII molecule which do have reduced binding to VWF or do not bind VWF at all.

Linker sequences

[0086] According to this invention, the therapeutic polypeptide moiety may be coupled to the HLEP moiety by a peptide linker. The linker should be non-immunogenic and may be a non-cleavable or cleavable linker.

[0087] Non-cleavable linkers may be comprised of alternating glycine and serine residues as exemplified in WO2007/090584.

[0088] In another embodiment of the invention the peptidic linker between the FVIII and/or the VWF moiety and the albumin moiety consists of peptide sequences, which serve as natural interdomain linkers in human proteins. Preferably such peptide sequences in their natural environment are located close to the protein surface and are accessible to the immune system so that one can assume a natural tolerance against this sequence. Examples are given in WO2007/090584.

[0089] Cleavable linkers should be flexible enough to allow cleavage by proteases. In a preferred embodiment the cleavage of the linker proceeds comparably fast as the activation of FVIII within the fusion protein, if the fusion protein is a modified FVIII.

[0090] The cleavable linker preferably comprises a sequence derived from

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

[0091] The linker region in a more preferred embodiment comprises a sequence of FVIII and/or VWF, which should result in a decreased risk of neoantigenic properties of the expressed fusion protein. Also in case the therapeutic protein is FVIII which needs to be proteolytically activated, the kinetics of the peptide linker cleavage will more closely reflect the coagulation-related activation kinetics of the zymogen.

[0092] In a preferred embodiment, the therapeutic polypeptide is FVIII zymogen and the HLEP is albumin. In this case the linker sequence is either derived from the sequences of the activation regions of FVIII, from the cleavage region of any substrate of FIX like FX or FVII or from the cleavage region of any substrate polypeptide that is cleaved by a protease in whose activation FIXa is involved.

[0093] In a highly preferred embodiment the linker peptide is derived from FVIII itself and

comprises of sequences encompassing the thrombin cleavage sites at amino acid positions 372, 740 and 1689 of SEQ ID NO. 15, respectively. In another preferred embodiment the linker peptide is derived from FX, FIX, FVII or FXI.

[0094] The linker peptides are preferably cleavable by the proteases of the coagulation system, for example FIIa, FIXa, FXa, FXIa, FXIIa and FVIIa.

[0095] Said linker sequences can also be used in the modified VWF of the invention.

[0096] Exemplary combinations of therapeutic polypeptide, cleavable linker and HLEP include the constructs listed in WO2007/090584 (for example in table 2 and figure 4) and WO2007/144173 (for example in table 3a and 3b), but are not limited to these.

Half-life enhancing polypeptides (HLEPs)

[0097] A "half-life enhancing polypeptide" as used herein is selected from the group consisting of albumin., It may be a full-length half-life-enhancing protein described herein or one or more fragments thereof that are capable of stabilizing or prolonging the therapeutic activity or the biological activity of the coagulation factor. Such fragments may be of 10 or more amino acids in length or may include at least about 15, at least about 20, at least about 25, at least about 30, at least about 50, at least about 100, or more contiguous amino acids from the HLEP sequence or may include part or all of specific domains of the respective HLEP, as long as the HLEP fragment provides a functional half-life extension of at least 25% compared to a wild-type FVIII or wild-type VWF.

[0098] The HLEP portion 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 FVIII or modified VWF.

[0099] In particular, the proposed FVIII HLEP or VWF HLEP fusion constructs of the invention may include naturally occurring polymorphic variants of HLEPs and fragments of HLEPs. The HLEP may be derived from any vertebrate, especially any mammal, for example human, monkey, cow, sheep, or pig. Non-mammalian HLEPs include, but are not limited to, hen and salmon.

Albumin as HLEP

[0100] 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).

[0101] As used herein, "albumin" refers collectively to albumin polypeptide or amino acid sequence, or an albumin fragment or variant, having one or more functional activities (e.g., biological activities) of albumin. In particular, "albumin" refers to human albumin or fragments thereof, especially the mature form of human albumin as shown in SEQ ID NO:16 herein or albumin from other vertebrates or fragments thereof, or analogs or variants of these molecules or fragments thereof.

[0102] In particular, the proposed FVIII fusion 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. The albumin variant may preferentially consist of or alternatively comprise at least one whole domain of albumin or fragments of said domains, for example domains 1 (amino acids 1-194 of SEQ ID NO:16), 2 (amino acids 195-387 of SEQ ID NO: 16), 3 (amino acids 388-585 of SEQ ID NO: 16), 1 + 2 (1-387 of SEQ ID NO: 16), 2 + 3 (195-585 of SEQ ID NO: 16) or 1 + 3 (amino acids 1-194 of SEQ ID NO: 16 + amino acids 388-585 of SEQ ID NO: 16). Each domain is itself made up of two homologous subdomains namely 1-105, 120-194, 195-291, 316-387, 388-491 and 512-585, with flexible inter-subdomain linker regions comprising residues Lys106 to Glu119, Glu292 to Val315 and Glu492 to Ala511.

[0103] The albumin portion of the proposed FVIII fusion constructs of the invention may comprise at least one subdomain or domain of HA or conservative modifications thereof.

Polynucleotides

[0104] The invention further relates to a polynucleotide encoding a modified coagulation factor, preferably a modified FVIII variant as described in this application. The term "polynucleotide(s)" generally refers to any polyribonucleotide or polydeoxyribonucleotide that may be unmodified RNA or DNA or modified RNA or DNA. The polynucleotide may be single- or double-stranded DNA, single or double-stranded RNA. As used herein, the term "polynucleotide(s)" also includes DNAs or RNAs that comprise one or more modified bases and/or unusual bases, such as inosine. It will be appreciated that a variety of modifications may be made to DNA and RNA that serve many useful purposes known to those of skill in the art. The term "polynucleotide(s)" as it is employed herein embraces such chemically, enzymatically or metabolically modified forms of polynucleotides, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including, for example, simple and complex cells.

[0105] The skilled person will understand that, due to the degeneracy of the genetic code, a given polypeptide can be encoded by different polynucleotides. These "variants" are encompassed by this invention.

[0106] Preferably, the polynucleotide of the invention is an isolated polynucleotide. The term "isolated" polynucleotide refers to a polynucleotide that is substantially free from other nucleic acid sequences, such as and not limited to other chromosomal and extrachromosomal DNA and RNA. Isolated polynucleotides may be purified from a host cell. Conventional nucleic acid purification methods known to skilled artisans may be used to obtain isolated polynucleotides. The term also includes recombinant polynucleotides and chemically synthesized polynucleotides.

[0107] The invention further relates to a group of polynucleotides which together encode the modified FVIII and/or the modified VWF of the invention. A first polynucleotide in the group may encode the N-terminal part of the modified FVIII, and a second polynucleotide may encode the C-terminal part of the modified FVIII.

[0108] Yet another aspect of the invention is a plasmid or vector comprising a polynucleotide according to the invention. Preferably, the plasmid or vector is an expression vector. In a particular embodiment, the vector is a transfer vector for use in human gene therapy.

[0109] The invention also relates to a group of plasmids or vectors that comprise the above group of polynucleotides. A first plasmid or vector may contain said first polynucleotide, and a second plasmid or vector may contain said second polynucleotide. By way of example, and with reference to coagulation factor FVIII, the coding sequences of the signal peptide, the A1 and A2 domains, the B domain sequence remainder and the HLEP may be cloned into the first expression vector and the coding sequences of A3, C1 and C2 with an appropriate signal peptide sequence may be cloned into the second expression vector. Both expression vectors are cotransfected into a suitable host cell, which will lead to the expression of the light and heavy chains of the FVIII molecule of the invention and the formation of a functional protein.

[0110] Alternatively, the coding sequence of the FVIII signal peptide, the A1 and A2 domains are cloned into the first expression vector and the coding sequences of the HLEP, FVIII A3, C1 and C2 with an appropriate signal peptide sequence are cloned into the second expression vector. Both expression vectors are cotransfected into a suitable host cell, which will lead to the expression of the light and heavy chains of the FVIII molecule of the invention and the formation of a functional protein.

[0111] Alternatively, both coding sequences are cloned into one expression vector either using two separate promoter sequences or one promoter and an internal ribosome entry site (IRES) element to direct the expression of both FVIII chains.

[0112] Still another aspect of the invention is a host cell comprising a polynucleotide, a plasmid or vector of the invention, or a group of polynucleotides or a group of plasmids or vectors as described herein.

[0113] The host cells of the invention may be employed in a method of producing a modified

coagulation factor, preferably a modified FVIII molecule, which is part of this invention. The method comprises:

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

[0114] It is preferred to purify the modified FVIII of the present invention to ≥ 80% purity, more preferably ≥ 95% purity, and particularly preferred is a pharmaceutically pure state that is greater than 99.9% pure with respect to contaminating macromolecules, particularly other proteins and nucleic acids, and free of infectious and pyrogenic agents. Preferably, an isolated or purified modified FVIII of the invention is substantially free of other, non-related polypeptides.

[0115] The various products of the invention are useful as medicaments. Accordingly, the invention relates to a pharmaceutical composition comprising a modified FVIII as described herein, a polynucleotide of the invention, or a plasmid or vector of the invention.

[0116] The invention also concerns a method of treating an individual suffering from a blood coagulation disorder such as hemophilia A or B. The method comprises administering to said individual an efficient amount of the FVIII or the complex of modified FVIII with non-modified VWF as described herein. In another embodiment, the method comprises administering to the individual an efficient amount of a polynucleotide of the invention or of a plasmid or vector of the invention. Alternatively, the method may comprise administering to the individual an efficient amount of the host cells of the invention described herein.

Expression of the proposed mutants

[0117] The production of recombinant mutant proteins at high levels in suitable host cells requires the assembly of the above-mentioned modified cDNAs into efficient transcriptional units together with suitable regulatory elements in a recombinant expression vector that can be propagated in various expression systems according to methods known to those skilled in the art. Efficient transcriptional regulatory elements could be derived from viruses having animal cells as their natural hosts or from the chromosomal DNA of animal cells. Preferably, promoter-enhancer combinations derived from the Simian Virus 40, adenovirus, BK polyoma virus, human cytomegalovirus, or the long terminal repeat of Rous sarcoma virus, or promoter-enhancer combinations including strongly constitutively transcribed genes in animal cells like beta-actin or GRP78 can be used. In order to achieve stable high levels of mRNA transcribed from the cDNAs, the transcriptional unit should contain in its 3'-proximal part a DNA region encoding a transcriptional termination-polyadenylation sequence. Preferably, this sequence is

derived from the Simian Virus 40 early transcriptional region, the rabbit beta-globin gene, or the human tissue plasminogen activator gene.

[0118] The cDNAs are then integrated into the genome of a suitable host cell line for expression of the modified FVIII and/or VWF proteins. Preferably this cell line should be an animal cell-line of vertebrate origin in order to ensure correct folding, disulfide bond formation, asparagine-linked glycosylation and other post-translational modifications as well as secretion into the cultivation medium. Examples on other post-translational modifications are tyrosine O-sulfation and proteolytic processing of the nascent polypeptide chain. Examples of cell lines that can be used are monkey COS-cells, mouse L-cells, mouse C127-cells, hamster BHK-21 cells, human embryonic kidney 293 cells, and hamster CHO-cells.

[0119] The recombinant expression vector encoding the corresponding cDNAs can be introduced into an animal cell line in several different ways. For instance, recombinant expression vectors can be created from vectors based on different animal viruses. Examples of these are vectors based on baculovirus, vaccinia virus, adenovirus, and preferably bovine papilloma virus.

[0120] The transcription units encoding the corresponding DNA's can also be introduced into animal cells together with another recombinant gene which may function as a dominant selectable marker in these cells in order to facilitate the isolation of specific cell clones which have integrated the recombinant DNA into their genome. Examples of this type of dominant selectable marker genes are Tn5 amino glycoside phosphotransferase, conferring resistance to geneticin (G418), hygromycin phosphotransferase, conferring resistance to hygromycin, and puromycin acetyl transferase, conferring resistance to puromycin. The recombinant expression vector encoding such a selectable marker can reside either on the same vector as the one encoding the cDNA of the desired protein, or it can be encoded on a separate vector which is simultaneously introduced and integrated to the genome of the host cell, frequently resulting in a tight physical linkage between the different transcription units.

[0121] Other types of selectable marker genes which can be used together with the cDNA of the desired protein are based on various transcription units encoding dihydrofolate reductase (dhfr). After introduction of this type of gene into cells lacking endogenous dhfr-activity, preferentially CHO-cells (DUKX-B11, DG-44), it will enable these to grow in media lacking nucleosides. An example of such a medium is Ham's F12 without hypoxanthine, thymidin, and glycine. These dhfr-genes can be introduced together with the FVIII cDNA transcriptional units into CHO-cells of the above type, either linked on the same vector or on different vectors, thus creating dhfr-positive cell lines producing recombinant protein.

[0122] If the above cell lines are grown in the presence of the cytotoxic dhfr-inhibitor methotrexate, new cell lines resistant to methotrexate will emerge. These cell lines may produce recombinant protein at an increased rate due to the amplified number of linked dhfr and the desired protein's transcriptional units. When propagating these cell lines in increasing concentrations of methotrexate (1-10000 nM), new cell lines can be obtained which produce

the desired protein at very high rate.

[0123] The above cell lines producing the desired protein can be grown on a large scale, either in suspension culture or on various solid supports. Examples of these supports are micro carriers based on dextran or collagen matrices, or solid supports in the form of hollow fibres or various ceramic materials. When grown in cell suspension culture or on micro carriers the culture of the above cell lines can be performed either as a bath culture or as a perfusion culture with continuous production of conditioned medium over extended periods of time. Thus, according to the present invention, the above cell lines are well suited for the development of an industrial process for the production of the desired recombinant mutant proteins

Purification and Formulation

[0124] The recombinant modified FVIII, which accumulates in the medium of secreting cells of the above types, can be concentrated and purified by a variety of biochemical and chromatographic methods, including methods utilizing differences in size, charge, hydrophobicity, solubility, specific affinity, etc. between the desired protein and other substances in the cell cultivation medium.

[0125] An example of such purification is the adsorption of the recombinant mutant protein to a monoclonal antibody, directed to e.g. a help human albumin, or directed to the respective coagulation factor, which is immobilised on a solid support. After adsorption of the modified FVIII to the support, washing and desorption, the protein can be further purified by a variety of chromatographic techniques based on the above properties. The order of the purification steps is chosen e.g. according to capacity and selectivity of the steps, stability of the support or other aspects. Preferred purification steps e.g. are but are not limited to ion exchange chromatography steps, immune affinity chromatography steps, affinity chromatography steps, hydrophobic interaction chromatography steps, dye chromatography steps, hydroxyapatite chromatography steps, multimodal chromatography steps, and size exclusion chromatography steps.

[0126] In order to minimize the theoretical risk of virus contaminations, additional steps may be included in the process that allow effective inactivation or elimination of viruses. Such steps e.g. are heat treatment in the liquid or solid state, treatment with solvents and/or detergents, radiation in the visible or UV spectrum, gamma-radiation or nanofiltration.

[0127] The modified polynucleotides (e.g. DNA) of this invention may also be integrated into a transfer vector for use in the human gene therapy.

[0128] The various embodiments described herein may be combined with each other. The present invention will be further described in more detail in the following examples thereof. This description of specific embodiments of the invention will be made in conjunction with the appended figures.

[0129] The modified FVIII as described in this invention can be formulated into pharmaceutical preparations for therapeutic use. The purified protein may be dissolved in conventional physiologically compatible aqueous buffer solutions to which there may be added, optionally, pharmaceutical excipients to provide pharmaceutical preparations.

[0130] Such pharmaceutical carriers and excipients as well as suitable pharmaceutical formulations are well known in the art (see for example "Pharmaceutical Formulation Development of Peptides and Proteins", Frokjaer et al., Taylor & Francis (2000) or "Handbook of Pharmaceutical Excipients", 3rd edition, Kibbe et al., Pharmaceutical Press (2000)). In particular, the pharmaceutical composition comprising the polypeptide variant of the invention may be formulated in lyophilized or stable liquid form. The polypeptide variant may be lyophilized by a variety of procedures known in the art. Lyophilized formulations are reconstituted prior to use by the addition of one or more pharmaceutically acceptable diluents such as sterile water for injection or sterile physiological saline solution.

[0131] Formulations of the composition are delivered to the individual by any pharmaceutically suitable means of administration. Various delivery systems are known and can be used to administer the composition by any convenient route. Preferentially, the compositions of the invention are administered systemically. For systemic use, insertion proteins of the invention are formulated for parenteral (e.g. intravenous, subcutaneous, intramuscular, intraperitoneal, intracerebral, intrapulmonar, intranasal or transdermal) or enteral (e.g., oral, vaginal or rectal) delivery according to conventional methods. The most preferential routes of administration are intravenous and subcutaneous administration. The formulations can be administered continuously by infusion or by bolus injection. Some formulations encompass slow release systems.

[0132] The insertion proteins of the present invention are administered to patients in a therapeutically effective dose, meaning a dose that is sufficient to produce the desired effects, preventing or lessening the severity or spread of the condition or indication being treated without reaching a dose which produces intolerable adverse side effects. The exact dose depends on many factors as e.g. the indication, formulation, mode of administration and has to be determined in preclinical and clinical trials for each respective indication.

[0133] The pharmaceutical composition of the invention may be administered alone or in conjunction with other therapeutic agents. These agents may be incorporated as part of the same pharmaceutical. One example of such an agent is the combination of modified FVIII with non-modified VWF.

Figures

[0134]

Figure 1: Antigen and activity levels of wild-type FVIII and FVIII-C-terminal albumin fusion polypeptides

Figure 2: Comparison of human FVIII:Ag pharmacokinetics in VWF ko mice following i.v. injection of 100 U (FVIII:Ag)/kg FVIII wildtype and FVIII-FP 1656 VWF (mean; n=4/timepoint)

Figure 3: VWF:RCo/VWF:Ag ratios of cell culture supernatants containing wt rVWF (1570/1212), rVWF-FP (1572/1212) containing C-terminally linked albumin, or a mixed expression cell culture containing a mixture of wt rVWF (1570/1212) and rVWF-FP (1572/1212) transfected in a ratio of 5:1. Values of about 0,8 were obtained in every case that are close to 1 which is the theoretical ratio of NHP according to the unit definitions.

Figure 4: SDS-Agarose gel electrophoresis of wild-type rVWF (1570/1212) expressed in HEK cells (B) and rVWF-FP (1572/1212) expressed also in HEK cells (A). Bands were detected using either antibodies to VWF or to albumin (HSA).

Figure 5: Comparison of human rWVF wildtype and rVWF-FP pharmacokinetics following i.v. injection of 100 IU VWF:Ag in rats (mean, n=2-3 /timepoint)

Examples:

Example 1: Generation of expression vectors for FVIII molecules with C-terminal albumin fusion

[0135] An expression plasmid based on pIRESpuro3 (BD Biosciences) containing the full length FVIII cDNA sequence in its multiple cloning site (pF8-FL) was first used to create a B domain deleted FVIII. For that oligonucleotides F8-1 and F8-2 (SEQ ID NO 1 and 2) were used in a site-directed mutagenesis experiment according to standard protocols (QuickChange XL Site Directed Mutagenesis Kit, Stratagene, La Jolla, CA, USA) using pF8-FL as a template to delete the B domain. In a second step a sequence encoding the amino acid sequence RRGR was introduced to connect R740 of the A2 domain with R1648 of the a3 domain. This was performed in another round of site-directed mutagenesis using primers F8-3 and F8-4 (SEQ ID NO 3 and 4). The resulting plasmid was called pF8-457.

[0136] A FVIII albumin fusion construct was generated stepwise. First, a PinAI cleavage site was introduced at the FVIII 3'terminus. For that a PCR fragment was generated using pF8-457 as template, using PCR primers We2827 and We2828 (SEQ ID NO 5 and 6), which was subsquently gel-purified, cut by restriction endonucleases BspE1 and NotI and ligated into pF8-457 previously digested with BspE1 and NotI. The resulting plasmid (pF8-1433) was then cut with enzymes PinAI and NotI and a fragment obtained by PCR on a human albumin cDNA containing plasmid using primers We 2829 and We 2830 (SEQ ID NO 7 and 8) and

subsequently digested with enzymes PinAI and NotI was inserted. The resulting expression plasmid (pF8-1434) contained the coding sequences for a B domain deleted FVIII followed by a PinAI site to insert linkers (encoding the amino acid sequence ThrGly) and the coding sequence for human albumin. The amino acid sequence encoded by pF8-1434 is depicted as SEQ ID NO 9.

[0137] Linker sequences separating the FVIII and albumin moieties could then easily be inserted into the newly created PinAI site described above. The insertion of two linker sequences is described in the following. In addition, based on pF8-1434, the TG linker might be deleted in completion and even deletions into the C-terminus of FVIII or the N-terminus of albumin can be performed using site directed mutagenesis.

[0138] Insertion of a cleavable linker, derived from the FVIII thrombin cleavage site: First a PCR fragment containing the sequence encoding the thrombin cleavage site at position 372 was generated by PCR using primers We2979 and We2980 (SEQ ID NO 10 and 11) and pF8-457 as template. This fragment was purified, digested with PinAI and ligated into PinAI digested pF8-1434. Sequencing verified insertion of correct orientation of the fragment, the resulting plasmid was called pF8-1563.

[0139] Insertion of a flexible glycine/serine linker: A PCR fragment containing the coding sequence for a 31 amino acid glycine/serine linker was amplified by PCR from pFVII-937 described in WO2007/090584 using primers We2991 and We2992 (SEQ ID NO 12 and 13). This fragment was then purified, digested by restriction endonuclease PinAI and ligated into PinAI digested pF8-1434. Sequencing verified insertion of correct orientation of the fragment, the resulting plasmid was called pF8-1568.

[0140] Using the protocols and plasmids described above and by applying molecular biology techniques known to those skilled in the art (and as described e.g. in Current Protocols in Molecular Biology, Ausubel FM et al. (eds.) John Wiley & Sons, Inc.; http://www.currentprotocols.com/WileyCDA/) other constructs can be made by the artisan to replace albumin by another HLEP or insert any other linker into the described PinAI site. Transfer of the FVIII/albumin cDNA into suitable vectors like pIRESneo3 (Invitrogen) and pEE12.4 (Lonza) permitted expression and selection of clones expressing the respective FVIII albumin fusion protein in CHO cells.

Example 2: Transfection and expression of FVIII and VWF proteins

[0141] Expression plasmids were grown up in E.coli TOP10 (Invitrogen, Carlsbad, CA, USA) and purified using standard protocols (Qiagen, Hilden, Germany). HEK-293 (Invitrogen) cells were transfected using the Lipofectamine 2000 reagent (Invitrogen) and grown up in serum-free medium (Invitrogen 293 Express) in the presence of 4 µg/ml Puromycin and optionally 0.5 IU/ml VWF. CHO cells (CHO-S, Invitrogen; CHOK1SV, Lonza) were transfected using the Lipofectamine 2000 reagent (Invitrogen) and grown up in serum-free medium (Invitrogen CD

CHO, 6 mM glutamine for CHO-S and CD-CHO for CHOK1SV) in the presence of 500-1000 µg/ml Geneticin (CHO-S only). For FVIII expression optionally 0.5 IU/ml VWF were added. For vWF expression an expression plasmid encoding PACE/furin (pFu-797) as described in WO2007/144173 was cotransfected. In another experiment two plasmids encoding VWF wild-type and VWF fused at the C-terminus to albumin were cotransfected with pFu-797 resulting in VWF multimeres with wild-type VWF monomers and albumin-fused VWF monomers (see figure 3). Transfected cell populations were spread through T-flasks into roller bottles or small scale fermenters from which supernatants were harvested for purification.

[0142] Table 2 lists HEK-293 expression data of the constructs described in example 1.

Table 2:

Construct	Activity [U/mL]
pF8-457	1.54
pF8-457 + 0.5 U/ml VWF	1.66
pF8-1434	1.59
pF8-1434 + 0.5 U/ml VWF	1.82
pF8-1563 + 0.5 U/ml VWF	2.04
pF8-1568 + 0.5 U/ml VWF	1.21

Example 3: Increased expression rate of FVIII albumin fusion protein

[0143] Figure 1 summarizes the results of an expression study of a FVIII albumin fusion protein in serum-free cell culture. HEK-293 cells were transfected in triplicate with pF8-1434 (FVIII C-terminal albumin fusion) and pF8-457 (FVIII wild-type), respectively, seeded into T80 flasks with equal cell numbers and grown in the absence of stabilizing VWF. Culture supernatant was then harvested after 96, 120 and 144 hours and tested for FVIII activity.

[0144] The results demonstrated an expression enhancing effect of the albumin moiety when present as an integral part of the FVIII molecule in cell culture. Consequently, the productivity was clearly improved in the case of the fusion protein compared to wild-type FVIII (Figure 1).

Example 4: Purification of FVIII proteins

[0145] To the expression supernatant containing the FVIII molecule a sufficient amount of an immune affinity resin was added to bind the FVIII activity almost completely. The immune affinity resin had been prepared by binding an appropriate anti-FVIII MAb covalently to Sephacryl S1000 resin used as a support. After washing of the resin it was filled into a chromatography column and washed again. Elution was done using a buffer containing 250

mM $CaCl_2$ and 50% ethylene glycol.

[0146] The immune affinity chromatography (IAC) fractions containing FVIII:C activity were pooled, dialyzed against formulation buffer (excipients: sodium chloride, sucrose, histidine, calcium chloride, and Tween 80), and concentrated. Samples were either stored frozen or freeze-dried using an appropriate freeze-drying cycle.

[0147] Alternatively, the FVIII containing cell culture supernatant is concentrated/purified by a first ion exchange chromatography followed by further purification using immune affinity chromatography (IAC). In this case the eluate of the ion exchange chromatography is loaded onto an IAC column using the above mentioned resin.

Example 5: Analysis of FVIII activity and antigen

[0148] For activity determination of FVIII:C in vitro either a clotting assay (e.g. Pathromtin SL reagent and FVIII deficient plasma delivered by Dade Behring, Germany) or a chromogenic assay (e.g. Coamatic FVIII:C assay delivered by Haemochrom) were used. The assays were performed according to the manufacturers instructions.

[0149] FVIII antigen (FVIII:Ag) was determined by an ELISA whose performance is known to those skilled in the art. Briefly, microplates were incubated with 100 µL per well of the capture antibody (sheep anti-human FVIII IgG, Cedarlane CL20035K-C, diluted 1:200 in Buffer A [Sigma C3041]) for 2 hours at ambient temperature. After washing plates three times with buffer B (Sigma P3563), serial dilutions of the test sample in sample diluent buffer (Cedarlane) as well as serial dilutions of a FVIII preparation (CSL Behring; 200 - 2 mU/mL) in sample diluent buffer (volumes per well: 100 µL) were incubated for two hours at ambient temperature. After three wash steps with buffer B, 100 µL of a 1:2 dilution in buffer B of the detection antibody (sheep anti-human FVIII IgG, Cedarlane CL20035K-D, peroxidase labelled) were added to each well and incubated for another hour at ambient temperature. After three wash steps with buffer B, 100 µL of substrate solution (1:10 (v/v) TMB OUVF : TMB Buffer OUVG, Dade Behring) were added per well and incubated for 30 minutes at ambient temperature in the dark. Addition of 100 µL stop solution (Dade Behring, OSFA) prepared the samples for reading in a suitable microplate reader at 450 nm wavelength. Concentrations of test samples were then calculated using the standard curve with the FVIII preparation as reference.

Example 6: Assessment of Pharmacokinetics of FVIII-FP in VWF ko mice following a single i.v. injection

[0150] In order to compare the pharmacokinetics of FVIII wildtype (DNA 457) and a C-terminal FVIII-FP (DNA 1656), both FVIII variants were administered intravenously to mice. A VWF ko mouse strain (Denis C. et al, Proc. Natl. Acad. Sci. USA, 1998, Vol 95, 9524-9529) was chosen

because, amongst other functions, VWF serves as a carrier and stabilizing protein for FVIII, thereby protecting FVIII from premature degradation, e.g. by proteases, and from premature elimination from circulation. For unmodified FVIII an undisturbed interaction with VWF is essential as exemplified by hemophilia A cases, caused by mutation in the C terminal region resulting in decreasing binding to VWF. In the case of modified FVIII such binding may, however, be even unwanted, in order to examine or achieve improved pharmacokinetics. Accordingly both products were injected i.v. at a dose of 100 U (FVIII:Ag)/kg as bolus to two groups of mice (Tab. 3). Blood was sampled retroorbitally at appropriate intervals starting at 5 minutes after application of the test substances and up to 24 hours. One blood sample / mouse was taken, processed to plasma and stored frozen at -20°C until analysis. Human FVIII:Ag concentration was quantified using an ELISA assay specific for human FVIII or by a mixed ELISA specific for human albumin and FVIII, respectively. The mean plasma concentration of the, for each timepoint pooled, samples was used for calculation of pharmacokinetic parameters. Half-live was calculated using the time points of the beta phase of elimination according to the formula $t_{1/2}$ = ln2 / k, whereas k is the slope of the regression line. The result is depicted in Figure 2. Surprisingly, FVIII-FP 1656 ($t_{1/2}$ = 3,06 h, between 5 and 960 min) had an about 3-4 times longer terminal half-life as compared to FVIII wildtype ($t_{1/2}$ = 0,8 h, between 5 and 240 min). In addition, the recovery of FVIII-FP 1656 was increased by about 20% as compared to wildtype FVIII (Tab. 4).

Table 3: Treatment groups for comparison of pharmacokinetics FVIII in VWF ko mice

Treatment	**Dose (FVIII:C) / volume / schedule / route**	**N**
FVIII wildtype	100 U (FVIII:Ag)/kg / 0.2 mL/20g b.w. / t=0 h /i.v..	24
FVIII-FP 1656	100 U(FVIII:Ag)/kg / 0.2 mL/20g b.w. / t=0 h /i.v..	24

Table 4: Bioavailability (%) of FVIII wildtype and modified FVIII, FVIII-FP 1656, upon i.v. injection into VWF ko mice

Treatment	**Bioavailability (%)**
FVIII wildtype	100
FVIII-FP 1656	120,4

Example 7: Generation of expression vectors for VWF wild-type and VWF albumin fusion proteins

[0151] An expression plasmid containing the full length VWF cDNA sequence in its multiple cloning site was generated first. For that the coding sequence of VWF was amplified by polymerase chain reaction (PCR) using primer set VWF+ and VWF- (SEQ ID NO. 17 and 18) under standard conditions known to those skilled in the art (and as described e.g. in Current Protocols in Molecular Biology, Ausubel FM et al. (eds.) John Wiley & Sons, Inc.;

http://www.currentprotocols.com/WileyCDA/) from a plasmid containing VWF cDNA (as obtainable commercially, e.g. pMT2-VWF from ATCC, No. 67122). The resulting PCR fragment was digested by restriction endonuclease EcoRI and ligated into expression vector pIRESpuro3 (BD Biosciences, Franklin Lakes, NJ, USA) which had been linearized by EcoRI. The resulting expression plasmid containing the wild-type cDNA of VWF downstream of the CMV promoter was called pVWF-1570.

[0152] A PCR fragment containing the coding sequence for a 31 amino acid glycine/serine linker and the human albumin cDNA was amplified from pFVII-937 described in WO2007/090584 using primers We2994 and We1335 (SEQ ID NO. 19 and 20). This PCR fragment was then digested by restriction endonuclease NotI and ligated into NotI digested pVWF-1570. The resulting plasmid containing the coding sequences of VWF wt, the linker sequence and human albumin was called pVWF-1574.

[0153] In order to achieve expression of a fusion protein several bases had to be deleted between VWF and the linker sequence. This was peformed by site directed mutagenesis according to standard protocols (QuickChange XL Site Directed Mutagenesis Kit, Stratagene, La Jolla, CA, USA) using oligonucleotides We2995 and We2996 (SEQ ID NO 21 and 22). The resulting expression plasmid called pVWF-1572 contained the coding sequences of VWF in frame with that of a 31 amino acid glycin/serine linker and human albumin. The amino acid sequence of the expressed rVWF-FP is outlined as SEQ ID No. 25. The amino acid sequence of the human VWF preproprotein is outlined as SEQ ID NO. 24.

[0154] Using the protocols and plasmids described above and by applying molecular biology techniques known to those skilled in the art (and as described e.g. in Current Protocols in Molecular Biology, ibid) other constructs can be made by the artisan for replacement of the albumin sequence by another HLEP sequence or the linker sequence by another linker sequence.

Example 8: Purification of VWF and VWF albumin fusion proteins

[0155] Cell culture supernatants containing VWF wild-type (rVWF wt) or VWF albumin fusion protein (rVWF-FP) were sterile-filtered through a 0,2µm filter and dialysed against equilibration buffer (EB; 10mM Tris-HCl, 10mM $CaCl_2$, pH 7.0). This material was then applied to a Heparin Fractogel column equilibrated with EB. The column was washed with EB and VWF proteins were eluated with 500mM NaCl in EB. The elution peak was concentrated and dialysed against FB buffer (3g/L sodium chloride, 20 g/L glycine, 5.5 g/L trisodium citrate dihydrate, pH 7.0). Finally the material was sterile filtrated and frozen in aliquots. If needed, further purification steps were applied comprising anion and/or cation exchange chromatography, HIC and SEC.

Example 9: Analysis of VWF activity and antigen

[0156] Samples were analysed by immunoturbidimetric determination of VWF:Ag (OPAB03, Siemens Healthcare Diagnostics, Marburg, Germany) and for collagen binding (Technozym VWF:CBA ELISA, Ref. 5450301 with calibrator set 5450310 and control set 5450312, Technoclone, Vienna, Austria) as described by the manufacturer.

[0157] VWF:RCo testing was done using the BC VWF reagent of Siemens Healthcare Diagnostics, Marburg, Germany according to the manufacturers description. The International Concentrate Standard was used as a primary standard preparation to calibrate an in-house standard preparation for day to day use.

[0158] The ratios of VWF:RCo and VWF:Ag assays are calculated in order to compare this parameter for different constructs tested. As is shown in figure 3 the VWF:RCo/VWF:Ag ratio was comparable for wt rVWF and the C-terminal rVWF-albumin fusion protein.

[0159] For pharmacokinetic analyses VWF antigen was determined by an ELISA whose performance is known to those skilled in the art. Briefly, microplates were incubated with 100 µL per well of the capture antibody (rabbit anti human VWF-IgG, Dako A0082 [Dako, Hamburg, Germany], diluted 1:2000 in buffer A [Sigma C3041, Sigma-Aldrich, Munich, Germany]) overnight at ambient temperature. After washing plates three times with buffer B (Sigma P3563), each well was incubated with 200 µL buffer C (Sigma P3688) for 1.5 hours at ambient temperature (blocking). After another three wash steps with buffer B, serial dilutions of the test sample in buffer B as well as serial dilutions of standard human plasma (ORKL21; 20 - 0.2 mU/mL; Siemens Healthcare Diagnostics, Marburg, Germany) in buffer B (volumes per well: 100 µL) were incubated for 1.5 hours at ambient temperature. After three wash steps with buffer B, 100 µL of a 1:16000 dilution in buffer B of the detection antibody (rabbit anti human VWF-IgG, Dako P0226, peroxidase labelled) were added to each well and incubated for 1 hour at ambient temperature. After three wash steps with buffer B, 100 µL of substrate solution (OUVF, Siemens Healthcare Diagnostics) were added per well and incubated for 30 minutes at ambient temperature in the dark. Addition of 100 µL undiluted stop dilution (OSFA, Siemens Healthcare Diagnostics) prepared the samples for reading in a suitable microplate reader at 450 nm wavelength. Concentrations of the test samples were then calculated using the standard curve with standard human plasma as reference.

Example 10: Multimer analysis of VWF and VWF albumin fusion proteins

[0160] VWF Multimer analysis was performed by SDS-agarose gel electrophoresis as recently described (Tatewaki et al.,. Thromb. Res. 52: 23-32 (1988), and Metzner et al., Haemophilia 4 (Suppl. 3): 25-32 (1998)) with minor modifications. Briefly, after equilibration in running buffer ready to use 1% agarose mini gels (BioRad) were used to standardize the method as far as possible. Comparable amounts of VWF antigen were subjected to electrophoresis on the SDS-agarose gels. After Western blotting the VWF protein bands were detected using anti-VWF (DAKO, prod. No. 0854) or anti-albumin antibodies followed by alkaline phosphatase labelled

anti-IgG antibodies (SIGMA, prod. No. 1305) and colour reaction quantified by densitometry.

[0161] Using wild-type rVWF (1570/797) and rVWF-FP (1572/797) it could be demonstrated by Western blotting and detection using anti-albumin or anti VWF antibodies that rVWF-FP forms a regular multimer distribution detected both by anti-albumin and anti-VWF antibodies (Figure 4). This confirms that although every subunit of the multimeric VWF contains albumin, a regular VWF multimer pattern is formed. The albumin moiety obviously does neither inhibit the N-terminal dimerization nor the C-terminal multimerization of the VWF molecules.

Example 11: Assessment of pharmacokinetics of VWF and VWF albumin fusion protein in rats following a single i.v. injection

[0162] rVWF-FP and rVWF wt were administered intravenously to a total of 4 CD rats each. The dose was 100 U (VWF:Ag)/kg body weight, at an injection volume of 4 mL/kg. Blood samples were drawn retroorbitally at appropriate intervals starting at 5 minutes after application of the test substances, using an alternating sampling scheme, resulting in samples from 2 animals / timepoint (t=0, 5, 30, 90 min, 4h, 1d for subset Nr. 1 and 0, 15 min, 1, 2, 8 h and 2 d for subset Nr. 2). The scheme was designed to minimize potential effects of blood sampling on the plasma concentration to be quantified. Blood was processed to plasma and stored deep frozen until analysis. The VWF:Ag level in plasma was subsequently quantified by an ELISA as described in Example 9. The mean plasma concentration was used for calculation of pharmacokinetic parameters. Half-life was calculated using the time points of the beta phase of elimination according to the formula $t_{1/2}$ = ln2 / k, whereas k is the slope of the regression line.

[0163] The result is depicted in Figure 5 (n=2/timepoint; mean). The terminal half-lives were calculated to be 32.4 min. for the rVWF-FP and 2.6 min. for rVWF wt. Recovery was also improved for the rVWF-FP with 42.1% compared to 16.1% for rVWF wt.

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Gln Ser Asp Gln Glu Glu Ile Asp Tyr Asp Asp Thr Ile Ser Val Glu
        755                 760                 765

Met Lys Lys Glu Asp Phe Asp Ile Tyr Asp Glu Asp Glu Asn Gln Ser

    770                 775                 780

Pro Arg Ser Phe Gln Lys Lys Thr Arg His Tyr Phe Ile Ala Ala Val
785                 790                 795                 800

Glu Arg Leu Trp Asp Tyr Gly Met Ser Ser Ser Pro His Val Leu Arg
                805                 810                 815

Asn Arg Ala Gln Ser Gly Ser Val Pro Gln Phe Lys Lys Val Val Phe
            820                 825                 830

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

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Gly Met  Phe Leu Tyr Glu Tyr  Ala Arg Arg His Pro  Asp Tyr Ser
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Val Val  Leu Leu Leu Arg Leu  Ala Lys Thr Tyr Glu  Thr Thr Leu
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Glu Lys  Cys Cys Ala Ala Ala  Asp Pro His Glu Cys  Tyr Ala Lys
    1790                 1795                 1800

Val Phe  Asp Glu Phe Lys Pro  Leu Val Glu Glu Pro  Gln Asn Leu
    1805                 1810                 1815

Ile Lys  Gln Asn Cys Glu Leu  Phe Glu Gln Leu Gly  Glu Tyr Lys
    1820                 1825                 1830

Phe Gln  Asn Ala Leu Leu Val  Arg Tyr Thr Lys Lys  Val Pro Gln
    1835                 1840                 1845

Val Ser  Thr Pro Thr Leu Val  Glu Val Ser Arg Asn  Leu Gly Lys
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Val Gly  Ser Lys Cys Cys Lys  His Pro Glu Ala Lys  Arg Met Pro
    1865                 1870                 1875

Cys Ala  Glu Asp Tyr Leu Ser  Val Val Leu Asn Gln  Leu Cys Val
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Leu His  Glu Lys Thr Pro Val  Ser Asp Arg Val Thr  Lys Cys Cys
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Thr Glu  Ser Leu Val Asn Arg  Arg Pro Cys Phe Ser  Ala Leu Glu
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Val Asp  Glu Thr Tyr Val Pro  Lys Glu Phe Asn Ala  Glu Thr Phe
    1925                 1930                 1935

Thr Phe  His Ala Asp Ile Cys  Thr Leu Ser Glu Lys  Glu Arg Gln
    1940                 1945                 1950

Ile Lys  Lys Gln Thr Ala Leu  Val Glu Leu Val Lys  His Lys Pro
    1955                 1960                 1965

Lys Ala  Thr Lys Glu Gln Leu  Lys Ala Val Met Asp  Asp Phe Ala
    1970                 1975                 1980

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

Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr Gln Thr Leu
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His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly Lys Ser Trp
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Val Ile Gly Met Gly Thr Thr Pro Glu Val His Ser Ile Phe Leu Glu
            260                 265                 270

Gly His Thr Phe Leu Val Arg Asn His Arg Gln Ala Ser Leu Glu Ile
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Ser Pro Ile Thr Phe Leu Thr Ala Gln Thr Leu Leu Met Asp Leu Gly
    290                 295                 300

Gln Phe Leu Leu Phe Cys His Ile Ser Ser His Gln His Asp Gly Met
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Glu Ala Tyr Val Lys Val Asp Ser Cys Pro Glu Glu Pro Gln Leu Arg
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Met Lys Asn Asn Glu Glu Ala Glu Asp Tyr Asp Asp Asp Leu Thr Asp
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Ser Glu Met Asp Val Val Arg Phe Asp Asp Asp Asn Ser Pro Ser Phe
        355                 360                 365

Ile Gln Ile Arg Ser Val Ala Lys Lys His Pro Lys Thr Trp Val His
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Tyr Ile Ala Ala Glu Glu Glu Asp Trp Asp Tyr Ala Pro Leu Val Leu
385                 390                 395                 400

Ala Pro Asp Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn Asn Gly Pro
                405                 410                 415

Gln Arg Ile Gly Arg Lys Tyr Lys Lys Val Arg Phe Met Ala Tyr Thr
            420                 425                 430

Asp Glu Thr Phe Lys Thr Arg Glu Ala Ile Gln His Glu Ser Gly Ile
        435                 440                 445

Leu Gly Pro Leu Leu Tyr Gly Glu Val Gly Asp Thr Leu Leu Ile Ile
    450                 455                 460

Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn Ile Tyr Pro His Gly Ile
465                 470                 475                 480

Thr Asp Val Arg Pro Leu Tyr Ser Arg Arg Leu Pro Lys Gly Val Lys
                485                 490                 495

His Leu Lys Asp Phe Pro Ile Leu Pro Gly Glu Ile Phe Lys Tyr Lys
            500                 505                 510
```

```
Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys Ser Asp Pro Arg Cys
        515                 520                 525

Leu Thr Arg Tyr Tyr Ser Ser Phe Val Asn Met Glu Arg Asp Leu Ala
    530                 535                 540

Ser Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu Ser Val Asp
545                 550                 555                 560

Gln Arg Gly Asn Gln Ile Met Ser Asp Lys Arg Asn Val Ile Leu Phe
                565                 570                 575

Ser Val Phe Asp Glu Asn Arg Ser Trp Tyr Leu Thr Glu Asn Ile Gln
            580                 585                 590

Arg Phe Leu Pro Asn Pro Ala Gly Val Gln Leu Glu Asp Pro Glu Phe
        595                 600                 605

Gln Ala Ser Asn Ile Met His Ser Ile Asn Gly Tyr Val Phe Asp Ser
    610                 615                 620

Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp Tyr Ile Leu
625                 630                 635                 640

Ser Ile Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe Ser Gly Tyr
                645                 650                 655

Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr Leu Phe Pro
            660                 665                 670

Phe Ser Gly Glu Thr Val Phe Met Ser Met Glu Asn Pro Gly Leu Trp
        675                 680                 685

Ile Leu Gly Cys His Asn Ser Asp Phe Arg Asn Arg Gly Met Thr Ala
    690                 695                 700

Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr Gly Asp Tyr Tyr Glu
705                 710                 715                 720

Asp Ser Tyr Glu Asp Ile Ser Ala Tyr Leu Leu Ser Lys Asn Asn Ala
                725                 730                 735

Ile Glu Pro Arg Ser Phe Ser Gln Asn Ser Arg His Arg Ser Thr Arg
            740                 745                 750

Gln Lys Gln Phe Asn Ala Thr Thr Ile Pro Glu Asn Asp Ile Glu Lys
        755                 760                 765

Thr Asp Pro Trp Phe Ala His Arg Thr Pro Met Pro Lys Ile Gln Asn
    770                 775                 780

Val Ser Ser Ser Asp Leu Leu Met Leu Leu Arg Gln Ser Pro Thr Pro
785                 790                 795                 800
```

```
His Gly Leu Ser Leu Ser Asp Leu Gln Glu Ala Lys Tyr Glu Thr Phe
                805                 810                 815

Ser Asp Asp Pro Ser Pro Gly Ala Ile Asp Ser Asn Asn Ser Leu Ser
            820                 825                 830

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

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

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

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

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

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

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

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

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

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

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

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

Asn Ser  Pro Ser Val Trp Gln  Asn Ile Leu Glu Ser  Asp Thr Glu
    1025                1030                1035

Phe Lys  Lys Val Thr Pro Leu  Ile His Asp Arg Met  Leu Met Asp
    1040                1045                1050

Lys Asn  Ala Thr Ala Leu Arg  Leu Asn His Met Ser  Asn Lys Thr
    1055                1060                1065

Thr Ser  Ser Lys Asn Met Glu  Met Val Gln Gln Lys  Lys Glu Gly
    1070                1075                1080

Pro Ile  Pro Pro Asp Ala Gln  Asn Pro Asp Met Ser  Phe Phe Lys
    1085                1090                1095
```

```
Met Leu  Phe Leu Pro Glu Ser  Ala Arg Trp Ile Gln  Arg Thr His
    1100                 1105                 1110

Gly Lys  Asn Ser Leu Asn Ser  Gly Gln Gly Pro Ser  Pro Lys Gln
    1115                 1120                 1125

Leu Val  Ser Leu Gly Pro Glu  Lys Ser Val Glu Gly  Gln Asn Phe
    1130                 1135                 1140

Leu Ser  Glu Lys Asn Lys Val  Val Val Gly Lys Gly  Glu Phe Thr
    1145                 1150                 1155

Lys Asp  Val Gly Leu Lys Glu  Met Val Phe Pro Ser  Ser Arg Asn
    1160                 1165                 1170

Leu Phe  Leu Thr Asn Leu Asp  Asn Leu His Glu Asn  Asn Thr His
    1175                 1180                 1185

Asn Gln  Glu Lys Lys Ile Gln  Glu Glu Ile Glu Lys  Lys Glu Thr
    1190                 1195                 1200

Leu Ile  Gln Glu Asn Val Val  Leu Pro Gln Ile His  Thr Val Thr
    1205                 1210                 1215

Gly Thr  Lys Asn Phe Met Lys  Asn Leu Phe Leu Leu  Ser Thr Arg
    1220                 1225                 1230

Gln Asn  Val Glu Gly Ser Tyr  Asp Gly Ala Tyr Ala  Pro Val Leu
    1235                 1240                 1245

Gln Asp  Phe Arg Ser Leu Asn  Asp Ser Thr Asn Arg  Thr Lys Lys
    1250                 1255                 1260

His Thr  Ala His Phe Ser Lys  Lys Gly Glu Glu Glu  Asn Leu Glu
    1265                 1270                 1275

Gly Leu  Gly Asn Gln Thr Lys  Gln Ile Val Glu Lys  Tyr Ala Cys
    1280                 1285                 1290

Thr Thr  Arg Ile Ser Pro Asn  Thr Ser Gln Gln Asn  Phe Val Thr
    1295                 1300                 1305

Gln Arg  Ser Lys Arg Ala Leu  Lys Gln Phe Arg Leu  Pro Leu Glu
    1310                 1315                 1320

Glu Thr  Glu Leu Glu Lys Arg  Ile Ile Val Asp Asp  Thr Ser Thr
    1325                 1330                 1335

Gln Trp  Ser Lys Asn Met Lys  His Leu Thr Pro Ser  Thr Leu Thr
    1340                 1345                 1350

Gln Ile  Asp Tyr Asn Glu Lys  Glu Lys Gly Ala Ile  Thr Gln Ser
    1355                 1360                 1365

Pro Leu  Ser Asp Cys Leu Thr  Arg Ser His Ser Ile  Pro Gln Ala
    1370                 1375                 1380
```

```
Asn Arg  Ser Pro Leu Pro Ile  Ala Lys Val Ser Ser  Phe Pro Ser
    1385                 1390                 1395

Ile Arg  Pro Ile Tyr Leu Thr  Arg Val Leu Phe Gln  Asp Asn Ser
    1400                 1405                 1410

Ser His  Leu Pro Ala Ala Ser  Tyr Arg Lys Lys Asp  Ser Gly Val
    1415                 1420                 1425

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

Ser Leu  Ala Ile Leu Thr Leu  Glu Met Thr Gly Asp  Gln Arg Glu
    1445                 1450                 1455

Val Gly  Ser Leu Gly Thr Ser  Ala Thr Asn Ser Val  Thr Tyr Lys
    1460                 1465                 1470

Lys Val  Glu Asn Thr Val Leu  Pro Lys Pro Asp Leu  Pro Lys Thr
    1475                 1480                 1485

Ser Gly  Lys Val Glu Leu Leu  Pro Lys Val His Ile  Tyr Gln Lys

    1490                 1495                 1500

Asp Leu  Phe Pro Thr Glu Thr  Ser Asn Gly Ser Pro  Gly His Leu
    1505                 1510                 1515

Asp Leu  Val Glu Gly Ser Leu  Leu Gln Gly Thr Glu  Gly Ala Ile
    1520                 1525                 1530

Lys Trp  Asn Glu Ala Asn Arg  Pro Gly Lys Val Pro  Phe Leu Arg
    1535                 1540                 1545

Val Ala  Thr Glu Ser Ser Ala  Lys Thr Pro Ser Lys  Leu Leu Asp
    1550                 1555                 1560

Pro Leu  Ala Trp Asp Asn His  Tyr Gly Thr Gln Ile  Pro Lys Glu
    1565                 1570                 1575

Glu Trp  Lys Ser Gln Glu Lys  Ser Pro Glu Lys Thr  Ala Phe Lys
    1580                 1585                 1590

Lys Lys  Asp Thr Ile Leu Ser  Leu Asn Ala Cys Glu  Ser Asn His
    1595                 1600                 1605

Ala Ile  Ala Ala Ile Asn Glu  Gly Gln Asn Lys Pro  Glu Ile Glu
    1610                 1615                 1620

Val Thr  Trp Ala Lys Gln Gly  Arg Thr Glu Arg Leu  Cys Ser Gln
    1625                 1630                 1635

Asn Pro  Pro Val Leu Lys Arg  His Gln Arg Glu Ile  Thr Arg Thr
    1640                 1645                 1650

Thr Leu  Gln Ser Asp Gln Glu  Glu Ile Asp Tyr Asp  Asp Thr Ile
```

1655 1660 1665

Ser Val Glu Met Lys Lys Glu Asp Phe Asp Ile Tyr Asp Glu Asp
1670 1675 1680

Glu Asn Gln Ser Pro Arg Ser Phe Gln Lys Lys Thr Arg His Tyr
1685 1690 1695

Phe Ile Ala Ala Val Glu Arg Leu Trp Asp Tyr Gly Met Ser Ser
1700 1705 1710

Ser Pro His Val Leu Arg Asn Arg Ala Gln Ser Gly Ser Val Pro
1715 1720 1725

Gln Phe Lys Lys Val Val Phe Gln Glu Phe Thr Asp Gly Ser Phe
1730 1735 1740

Thr Gln Pro Leu Tyr Arg Gly Glu Leu Asn Glu His Leu Gly Leu
1745 1750 1755

Leu Gly Pro Tyr Ile Arg Ala Glu Val Glu Asp Asn Ile Met Val
1760 1765 1770

Thr Phe Arg Asn Gln Ala Ser Arg Pro Tyr Ser Phe Tyr Ser Ser
1775 1780 1785

Leu Ile Ser Tyr Glu Glu Asp Gln Arg Gln Gly Ala Glu Pro Arg
1790 1795 1800

Lys Asn Phe Val Lys Pro Asn Glu Thr Lys Thr Tyr Phe Trp Lys
1805 1810 1815

Val Gln His His Met Ala Pro Thr Lys Asp Glu Phe Asp Cys Lys
1820 1825 1830

Ala Trp Ala Tyr Phe Ser Asp Val Asp Leu Glu Lys Asp Val His
1835 1840 1845

Ser Gly Leu Ile Gly Pro Leu Leu Val Cys His Thr Asn Thr Leu
1850 1855 1860

Asn Pro Ala His Gly Arg Gln Val Thr Val Gln Glu Phe Ala Leu
1865 1870 1875

Phe Phe Thr Ile Phe Asp Glu Thr Lys Ser Trp Tyr Phe Thr Glu
1880 1885 1890

Asn Met Glu Arg Asn Cys Arg Ala Pro Cys Asn Ile Gln Met Glu
1895 1900 1905

Asp Pro Thr Phe Lys Glu Asn Tyr Arg Phe His Ala Ile Asn Gly
1910 1915 1920

Tyr Ile Met Asp Thr Leu Pro Gly Leu Val Met Ala Gln Asp Gln
1925 1930 1935

Arg Ile Arg Trp Tyr Leu Leu Ser Met Gly Ser Asn Glu Asn Ile

1940 1945 1950

His Ser Ile His Phe Ser Gly His Val Phe Thr Val Arg Lys Lys
1955 1960 1965

Glu Glu Tyr Lys Met Ala Leu Tyr Asn Leu Tyr Pro Gly Val Phe
1970 1975 1980

Glu Thr Val Glu Met Leu Pro Ser Lys Ala Gly Ile Trp Arg Val
1985 1990 1995

Glu Cys Leu Ile Gly Glu His Leu His Ala Gly Met Ser Thr Leu
2000 2005 2010

Phe Leu Val Tyr Ser Asn Lys Cys Gln Thr Pro Leu Gly Met Ala
2015 2020 2025

Ser Gly His Ile Arg Asp Phe Gln Ile Thr Ala Ser Gly Gln Tyr
2030 2035 2040

Gly Gln Trp Ala Pro Lys Leu Ala Arg Leu His Tyr Ser Gly Ser
2045 2050 2055

Ile Asn Ala Trp Ser Thr Lys Glu Pro Phe Ser Trp Ile Lys Val
2060 2065 2070

Asp Leu Leu Ala Pro Met Ile Ile His Gly Ile Lys Thr Gln Gly
2075 2080 2085

Ala Arg Gln Lys Phe Ser Ser Leu Tyr Ile Ser Gln Phe Ile Ile
2090 2095 2100

Met Tyr Ser Leu Asp Gly Lys Lys Trp Gln Thr Tyr Arg Gly Asn
2105 2110 2115

Ser Thr Gly Thr Leu Met Val Phe Phe Gly Asn Val Asp Ser Ser
2120 2125 2130

Gly Ile Lys His Asn Ile Phe Asn Pro Pro Ile Ile Ala Arg Tyr
2135 2140 2145

Ile Arg Leu His Pro Thr His Tyr Ser Ile Arg Ser Thr Leu Arg
2150 2155 2160

Met Glu Leu Met Gly Cys Asp Leu Asn Ser Cys Ser Met Pro Leu
2165 2170 2175

Gly Met Glu Ser Lys Ala Ile Ser Asp Ala Gln Ile Thr Ala Ser
2180 2185 2190

Ser Tyr Phe Thr Asn Met Phe Ala Thr Trp Ser Pro Ser Lys Ala
2195 2200 2205

Arg Leu His Leu Gln Gly Arg Ser Asn Ala Trp Arg Pro Gln Val
2210 2215 2220

Asn Asn Pro Lys Glu Trp Leu Gln Val Asp Phe Gln Lys Thr Met
2225 2230 2235

```
Lys Val  Thr Gly Val Thr Thr  Gln Gly Val Lys Ser  Leu Leu Thr
    2240                 2245                 2250

Ser Met  Tyr Val Lys Glu Phe  Leu Ile Ser Ser Ser  Gln Asp Gly
    2255                 2260                 2265

His Gln  Trp Thr Leu Phe Phe  Gln Asn Gly Lys Val  Lys Val Phe
    2270                 2275                 2280

Gln Gly  Asn Gln Asp Ser Phe  Thr Pro Val Val Asn  Ser Leu Asp
    2285                 2290                 2295

Pro Pro  Leu Leu Thr Arg Tyr  Leu Arg Ile His Pro  Gln Ser Trp
    2300                 2305                 2310

Val His  Gln Ile Ala Leu Arg  Met Glu Val Leu Gly  Cys Glu Ala
    2315                 2320                 2325

Gln Asp  Leu Tyr
    2330
```

<210> 16

<211> 585

<212> PRT

<213> Homo sapiens

<400> 16

```
Asp Ala His Lys Ser Glu Val Ala His Arg Phe Lys Asp Leu Gly Glu
1               5                  10                  15

Glu Asn Phe Lys Ala Leu Val Leu Ile Ala Phe Ala Gln Tyr Leu Gln
            20                  25                  30

Gln Cys Pro Phe Glu Asp His Val Lys Leu Val Asn Glu Val Thr Glu
        35                  40                  45

Phe Ala Lys Thr Cys Val Ala Asp Glu Ser Ala Glu Asn Cys Asp Lys
    50                  55                  60

Ser Leu His Thr Leu Phe Gly Asp Lys Leu Cys Thr Val Ala Thr Leu
65                  70                  75                  80

Arg Glu Thr Tyr Gly Glu Met Ala Asp Cys Cys Ala Lys Gln Glu Pro
                85                  90                  95

Glu Arg Asn Glu Cys Phe Leu Gln His Lys Asp Asp Asn Pro Asn Leu
            100                 105                 110

Pro Arg Leu Val Arg Pro Glu Val Asp Val Met Cys Thr Ala Phe His
        115                 120                 125

Asp Asn Glu Glu Thr Phe Leu Lys Lys Tyr Leu Tyr Glu Ile Ala Arg
    130                 135                 140
```

```
Arg His Pro Tyr Phe Tyr Ala Pro Glu Leu Leu Phe Phe Ala Lys Arg
145                 150                 155                 160

Tyr Lys Ala Ala Phe Thr Glu Cys Cys Gln Ala Ala Asp Lys Ala Ala
                165                 170                 175

Cys Leu Leu Pro Lys Leu Asp Glu Leu Arg Asp Glu Gly Lys Ala Ser
            180                 185                 190

Ser Ala Lys Gln Arg Leu Lys Cys Ala Ser Leu Gln Lys Phe Gly Glu
        195                 200                 205

Arg Ala Phe Lys Ala Trp Ala Val Ala Arg Leu Ser Gln Arg Phe Pro
    210                 215                 220

Lys Ala Glu Phe Ala Glu Val Ser Lys Leu Val Thr Asp Leu Thr Lys
225                 230                 235                 240

Val His Thr Glu Cys Cys His Gly Asp Leu Leu Glu Cys Ala Asp Asp
                245                 250                 255

Arg Ala Asp Leu Ala Lys Tyr Ile Cys Glu Asn Gln Asp Ser Ile Ser
            260                 265                 270

Ser Lys Leu Lys Glu Cys Cys Glu Lys Pro Leu Leu Glu Lys Ser His
        275                 280                 285

Cys Ile Ala Glu Val Glu Asn Asp Glu Met Pro Ala Asp Leu Pro Ser
    290                 295                 300

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

Glu Ala Lys Asp Val Phe Leu Gly Met Phe Leu Tyr Glu Tyr Ala Arg

                325                 330                 335

Arg His Pro Asp Tyr Ser Val Val Leu Leu Leu Arg Leu Ala Lys Thr
            340                 345                 350

Tyr Glu Thr Thr Leu Glu Lys Cys Cys Ala Ala Ala Asp Pro His Glu
        355                 360                 365

Cys Tyr Ala Lys Val Phe Asp Glu Phe Lys Pro Leu Val Glu Glu Pro
    370                 375                 380

Gln Asn Leu Ile Lys Gln Asn Cys Glu Leu Phe Glu Gln Leu Gly Glu
385                 390                 395                 400

Tyr Lys Phe Gln Asn Ala Leu Leu Val Arg Tyr Thr Lys Lys Val Pro
                405                 410                 415

Gln Val Ser Thr Pro Thr Leu Val Glu Val Ser Arg Asn Leu Gly Lys
            420                 425                 430

Val Gly Ser Lys Cys Cys Lys His Pro Glu Ala Lys Arg Met Pro Cys
```

435 440 445

Ala Glu Asp Tyr Leu Ser Val Val Leu Asn Gln Leu Cys Val Leu His
450 455 460

Glu Lys Thr Pro Val Ser Asp Arg Val Thr Lys Cys Cys Thr Glu Ser
465 470 475 480

Leu Val Asn Arg Arg Pro Cys Phe Ser Ala Leu Glu Val Asp Glu Thr
485 490 495

Tyr Val Pro Lys Glu Phe Asn Ala Glu Thr Phe Thr Phe His Ala Asp
500 505 510

Ile Cys Thr Leu Ser Glu Lys Glu Arg Gln Ile Lys Lys Gln Thr Ala
515 520 525

Leu Val Glu Leu Val Lys His Lys Pro Lys Ala Thr Lys Glu Gln Leu
530 535 540

Lys Ala Val Met Asp Asp Phe Ala Ala Phe Val Glu Lys Cys Cys Lys
545 550 555 560

Ala Asp Asp Lys Glu Thr Cys Phe Ala Glu Glu Gly Lys Lys Leu Val
565 570 575

Ala Ala Ser Gln Ala Ala Leu Gly Leu
580 585

<210> 17
<211> 30
<212> DNA
<213> Artificial

<220>
<223> Primer

<400> 17
ttcgaattcc cgcagccctc atttgcaggg 30

<210> 18
<211> 31
<212> DNA
<213> Artificial

<220>
<223> Primer

<400> 18
tccgaattcc ggcagcagca ggcacccatg c 31

<210> 19
<211> 25

<212> DNA
<213> Artificial

<220>
<223> Primer

<400> 19
gcggcggccg cgagccccat ttccc 25

<210> 20
<211> 18
<212> DNA
<213> Artificial

<220>
<223> Primer

<400> 20
gagagggagt actcaccc 18

<210> 21
<211> 27
<212> DNA
<213> Artificial

<220>
<223> Primer

<400> 21
ggaagtgcag caagtcgagc gggggat 27

<210> 22
<211> 27
<212> DNA
<213> Artificial

<220>
<223> Primer

<400> 22
atcccccgct cgacttgctg cacttcc 27

<210> 23
<211> 585
<212> PRT
<213> Homo sapiens

<400> 23
Asp Ala His Lys Ser Glu Val Ala His Arg Phe Lys Asp Leu Gly Glu

```
1               5                  10                  15

Glu Asn Phe Lys Ala Leu Val Leu Ile Ala Phe Ala Gln Tyr Leu Gln
            20                  25                  30

Gln Cys Pro Phe Glu Asp His Val Lys Leu Val Asn Glu Val Thr Glu
        35                  40                  45

Phe Ala Lys Thr Cys Val Ala Asp Glu Ser Ala Glu Asn Cys Asp Lys
    50                  55                  60

Ser Leu His Thr Leu Phe Gly Asp Lys Leu Cys Thr Val Ala Thr Leu
65                  70                  75                  80

Arg Glu Thr Tyr Gly Glu Met Ala Asp Cys Cys Ala Lys Gln Glu Pro
                85                  90                  95

Glu Arg Asn Glu Cys Phe Leu Gln His Lys Asp Asp Asn Pro Asn Leu
            100                 105                 110

Pro Arg Leu Val Arg Pro Glu Val Asp Val Met Cys Thr Ala Phe His
        115                 120                 125

Asp Asn Glu Glu Thr Phe Leu Lys Lys Tyr Leu Tyr Glu Ile Ala Arg
    130                 135                 140

Arg His Pro Tyr Phe Tyr Ala Pro Glu Leu Leu Phe Phe Ala Lys Arg
145                 150                 155                 160

Tyr Lys Ala Ala Phe Thr Glu Cys Cys Gln Ala Ala Asp Lys Ala Ala
                165                 170                 175

Cys Leu Leu Pro Lys Leu Asp Glu Leu Arg Asp Glu Gly Lys Ala Ser
            180                 185                 190

Ser Ala Lys Gln Arg Leu Lys Cys Ala Ser Leu Gln Lys Phe Gly Glu
        195                 200                 205

Arg Ala Phe Lys Ala Trp Ala Val Ala Arg Leu Ser Gln Arg Phe Pro
    210                 215                 220

Lys Ala Glu Phe Ala Glu Val Ser Lys Leu Val Thr Asp Leu Thr Lys
225                 230                 235                 240

Val His Thr Glu Cys Cys His Gly Asp Leu Leu Glu Cys Ala Asp Asp
                245                 250                 255

Arg Ala Asp Leu Ala Lys Tyr Ile Cys Glu Asn Gln Asp Ser Ile Ser
            260                 265                 270

Ser Lys Leu Lys Glu Cys Cys Glu Lys Pro Leu Leu Glu Lys Ser His
        275                 280                 285

Cys Ile Ala Glu Val Glu Asn Asp Glu Met Pro Ala Asp Leu Pro Ser
    290                 295                 300
```

```
Leu Ala Ala Asp Phe Val Glu Ser Lys Asp Val Cys Lys Asn Tyr Ala
305                 310                 315                 320

Glu Ala Lys Asp Val Phe Leu Gly Met Phe Leu Tyr Glu Tyr Ala Arg
                325                 330                 335

Arg His Pro Asp Tyr Ser Val Val Leu Leu Leu Arg Leu Ala Lys Thr
            340                 345                 350

Tyr Glu Thr Thr Leu Glu Lys Cys Cys Ala Ala Ala Asp Pro His Glu
        355                 360                 365

Cys Tyr Ala Lys Val Phe Asp Glu Phe Lys Pro Leu Val Glu Glu Pro
    370                 375                 380

Gln Asn Leu Ile Lys Gln Asn Cys Glu Leu Phe Glu Gln Leu Gly Glu
385                 390                 395                 400

Tyr Lys Phe Gln Asn Ala Leu Leu Val Arg Tyr Thr Lys Lys Val Pro
                405                 410                 415

Gln Val Ser Thr Pro Thr Leu Val Glu Val Ser Arg Asn Leu Gly Lys
            420                 425                 430

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

Ala Glu Asp Tyr Leu Ser Val Val Leu Asn Gln Leu Cys Val Leu His
    450                 455                 460

Glu Lys Thr Pro Val Ser Asp Arg Val Thr Lys Cys Cys Thr Glu Ser
465                 470                 475                 480

Leu Val Asn Arg Arg Pro Cys Phe Ser Ala Leu Glu Val Asp Glu Thr
                485                 490                 495

Tyr Val Pro Lys Glu Phe Asn Ala Glu Thr Phe Thr Phe His Ala Asp
            500                 505                 510

Ile Cys Thr Leu Ser Glu Lys Glu Arg Gln Ile Lys Lys Gln Thr Ala
        515                 520                 525

Leu Val Glu Leu Val Lys His Lys Pro Lys Ala Thr Lys Glu Gln Leu
    530                 535                 540

Lys Ala Val Met Asp Asp Phe Ala Ala Phe Val Glu Lys Cys Cys Lys
545                 550                 555                 560

Ala Asp Asp Lys Glu Thr Cys Phe Ala Glu Glu Gly Lys Lys Leu Val
                565                 570                 575

Ala Ala Ser Gln Ala Ala Leu Gly Leu
            580                 585
```

<210> 24

<211> 2813

<212> PRT

<213> homo sapiens

<400> 24

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Met Ile Pro Ala Arg Phe Ala Gly Val Leu Leu Ala Leu Ala Leu Ile
1               5                   10                  15

Leu Pro Gly Thr Leu Cys Ala Glu Gly Thr Arg Gly Arg Ser Ser Thr
            20                  25                  30

Ala Arg Cys Ser Leu Phe Gly Ser Asp Phe Val Asn Thr Phe Asp Gly
        35                  40                  45

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

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

Arg Val Ser Leu Ser Val Tyr Leu Gly Glu Phe Phe Asp Ile His Leu
                85                  90                  95

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

Tyr Ala Ser Lys Gly Leu Tyr Leu Glu Thr Glu Ala Gly Tyr Tyr Lys
        115                 120                 125

Leu Ser Gly Glu Ala Tyr Gly Phe Val Ala Arg Ile Asp Gly Ser Gly
    130                 135                 140

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

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

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

Leu Ser Ser Gly Glu Gln Trp Cys Glu Arg Ala Ser Pro Pro Ser Ser
        195                 200                 205

Ser Cys Asn Ile Ser Ser Gly Glu Met Gln Lys Gly Leu Trp Glu Gln
    210                 215                 220

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

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

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

Arg Thr Cys Ala Gln Glu Gly Met Val Leu Tyr Gly Trp Thr Asp His
```

```
        275                 280                 285

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

Val Ser Pro Cys Ala Arg Thr Cys Gln Ser Leu His Ile Asn Glu Met

305                 310                 315                 320

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

Leu Asp Glu Gly Leu Cys Val Glu Ser Thr Glu Cys Pro Cys Val His
            340                 345                 350

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

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

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

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

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

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

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

Ala Met Asp Gly Gln Asp Ile Gln Leu Pro Leu Leu Lys Gly Asp Leu
465                 470                 475                 480

Arg Ile Gln His Thr Val Thr Ala Ser Val Arg Leu Ser Tyr Gly Glu
                485                 490                 495

Asp Leu Gln Met Asp Trp Asp Gly Arg Gly Arg Leu Leu Val Lys Leu
            500                 505                 510

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

Gly Asn Gln Gly Asp Asp Phe Leu Thr Pro Ser Gly Leu Ala Glu Pro
    530                 535                 540

Arg Val Glu Asp Phe Gly Asn Ala Trp Lys Leu His Gly Asp Cys Gln
545                 550                 555                 560

Asp Leu Gln Lys Gln His Ser Asp Pro Cys Ala Leu Asn Pro Arg Met
                565                 570                 575
```

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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Leu Lys Tyr Leu Phe Pro Gly Glu Cys Gln Tyr Val Leu Val Gln Asp
                885                 890                 895

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

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

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

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

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

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

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

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

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

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

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

Gln Asp  Cys Asn Lys Leu Val  Asp Pro Glu Pro Tyr  Leu Asp Val
    1070                1075                1080

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Glu Val  Leu Lys Ala Phe Val  Val Asp Met Met Glu  Arg Leu Arg

     1295                 1300                 1305

Ile Ser  Gln Lys Trp Val Arg  Val Ala Val Val Glu  Tyr His Asp
     1310                 1315                 1320

Gly Ser  His Ala Tyr Ile Gly  Leu Lys Asp Arg Lys  Arg Pro Ser
     1325                 1330                 1335

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

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

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

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

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

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

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

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

Asp Leu  Ala Pro Glu Ala Pro  Pro Pro Thr Leu Pro  Pro Asp Met
    1460                 1465                 1470

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

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

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

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

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

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

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

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

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

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

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

Leu Glu  Arg Ile Gly Trp Pro  Asn Ala Pro Ile Leu  Ile Gln Asp
    1640                 1645                 1650

Phe Glu  Thr Leu Pro Arg Glu  Ala Pro Asp Leu Val  Leu Gln Arg
    1655                 1660                 1665

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

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

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

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

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

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

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

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

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

Ser Val  Asp Ser Val Asp Ala  Ala Ala Asp Ala Ala  Arg Ser Asn
    1805                 1810                 1815

Arg Val  Thr Val Phe Pro Ile  Gly Ile Gly Asp Arg  Tyr Asp Ala
    1820                 1825                 1830

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

Val Lys  Leu Gln Arg Ile Glu  Asp Leu Pro Thr Met  Val Thr Leu
    1850                 1855                 1860

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

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

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

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

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

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

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

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

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

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

Val Glu  Leu His Ser Asp Met  Glu Val Thr Val Asn  Gly Arg Leu
```

```
    2015                2020                2025

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Pro Asp  Lys Val Met Leu Glu  Gly Ser Cys Val Pro  Glu Glu Ala

    2240                2245                2250

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

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

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

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

Gln Asn  Ala Asp Gln Cys Cys  Pro Glu Tyr Glu Cys  Val Cys Asp
    2315                 2320                 2325

Pro Val  Ser Cys Asp Leu Pro  Pro Val Pro His Cys  Glu Arg Gly
    2330                 2335                 2340

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Ala Cys  Cys Pro Ser Cys Arg  Cys Glu Arg Met Glu  Ala Cys Met
    2570                 2575                 2580
```

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

Val Cys  Thr Thr Cys Arg Cys  Met Val Gln Val Gly  Val Ile Ser
    2600                 2605                 2610

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

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

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

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

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

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

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

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

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

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

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

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

Val Tyr  His Glu Val Leu Asn  Ala Met Glu Cys Lys  Cys Ser Pro
    2795                 2800                 2805

Arg Lys  Cys Ser Lys
    2810
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<210> 25

<211> 3429

<212> PRT

<213> Artificial

<220>

<223> Amino acid sequence of human VWF albumin fusion preproprotein

<400> 25

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Met Ile Pro Ala Arg Phe Ala Gly Val Leu Leu Ala Leu Ala Leu Ile
1               5                  10                  15

Leu Pro Gly Thr Leu Cys Ala Glu Gly Thr Arg Gly Arg Ser Ser Thr
            20                  25                  30

Ala Arg Cys Ser Leu Phe Gly Ser Asp Phe Val Asn Thr Phe Asp Gly
        35                  40                  45

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

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

Arg Val Ser Leu Ser Val Tyr Leu Gly Glu Phe Phe Asp Ile His Leu
                85                  90                  95

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

Tyr Ala Ser Lys Gly Leu Tyr Leu Glu Thr Glu Ala Gly Tyr Tyr Lys
        115                 120                 125

Leu Ser Gly Glu Ala Tyr Gly Phe Val Ala Arg Ile Asp Gly Ser Gly
    130                 135                 140

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

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

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

Leu Ser Ser Gly Glu Gln Trp Cys Glu Arg Ala Ser Pro Pro Ser Ser
        195                 200                 205

Ser Cys Asn Ile Ser Ser Gly Glu Met Gln Lys Gly Leu Trp Glu Gln
    210                 215                 220

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

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

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

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

```
Ser Ala Cys Ser Pro Val Cys Pro Ala Gly Met Glu Tyr Arg Gln Cys
    290                 295                 300

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

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

Leu Asp Glu Gly Leu Cys Val Glu Ser Thr Glu Cys Pro Cys Val His
            340                 345                 350

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

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

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

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

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

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

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

Ala Met Asp Gly Gln Asp Ile Gln Leu Pro Leu Leu Lys Gly Asp Leu
465                 470                 475                 480

Arg Ile Gln His Thr Val Thr Ala Ser Val Arg Leu Ser Tyr Gly Glu
                485                 490                 495

Asp Leu Gln Met Asp Trp Asp Gly Arg Gly Arg Leu Leu Val Lys Leu
            500                 505                 510

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

Gly Asn Gln Gly Asp Asp Phe Leu Thr Pro Ser Gly Leu Ala Glu Pro
    530                 535                 540

Arg Val Glu Asp Phe Gly Asn Ala Trp Lys Leu His Gly Asp Cys Gln
545                 550                 555                 560

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Cys Val Cys Arg Asp Arg Lys Trp Asn Cys Thr Asp His Val Cys Asp

    850                 855                 860

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

Leu Lys Tyr Leu Phe Pro Gly Glu Cys Gln Tyr Val Leu Val Gln Asp
                885                 890                 895
```

885 890 895

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

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

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

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

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

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

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

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

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

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

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

Gln Asp Cys Asn Lys Leu Val Asp Pro Glu Pro Tyr Leu Asp Val
1070 1075 1080

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

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

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

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

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

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

His Ala His Cys Pro Pro Gly Lys Ile Leu Asp Glu Leu Leu Gln

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    1175                1180                1185

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

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

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

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

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

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

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

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

Ile Ser  Gln Lys Trp Val Arg  Val Ala Val Val Glu  Tyr His Asp
    1310                1315                1320

Gly Ser  His Ala Tyr Ile Gly  Leu Lys Asp Arg Lys  Arg Pro Ser
    1325                1330                1335

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

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

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

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

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

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

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

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

Asp Leu  Ala Pro Glu Ala Pro  Pro Pro Thr Leu Pro  Pro Asp Met
    1460                1465                1470
```

1460 1465 1470

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

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

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

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

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

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

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

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

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

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

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

Leu Glu Arg Ile Gly Trp Pro Asn Ala Pro Ile Leu Ile Gln Asp
1640 1645 1650

Phe Glu Thr Leu Pro Arg Glu Ala Pro Asp Leu Val Leu Gln Arg
1655 1660 1665

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

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

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

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

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

Pro Trp Asn Val Val Pro Glu Lys Ala His Leu Leu Ser Leu Val

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    1745                1750                1755

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

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

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

Ser Val  Asp Ser Val Asp Ala  Ala Ala Asp Ala Ala  Arg Ser Asn

    1805                1810                1815

Arg Val  Thr Val Phe Pro Ile  Gly Ile Gly Asp Arg  Tyr Asp Ala
    1820                1825                1830

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

Val Lys  Leu Gln Arg Ile Glu  Asp Leu Pro Thr Met  Val Thr Leu
    1850                1855                1860

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

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

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

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

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

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

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

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

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

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

Val Glu  Leu His Ser Asp Met  Glu Val Thr Val Asn  Gly Arg Leu
    2015                2020                2025
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Val Ser  Val Pro Tyr Val Gly  Gly Asn Met Glu Val  Asn Val Tyr
    2030                 2035                 2040

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Ala Lys  Ala Pro Thr Cys Gly  Leu Cys Glu Val Ala  Arg Leu Arg
    2300                 2305                 2310
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Gln Asn  Ala Asp Gln Cys Cys  Pro Glu Tyr Glu Cys  Val Cys Asp
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Pro Val  Ser Cys Asp Leu Pro  Pro Val Pro His Cys  Glu Arg Gly
    2330                 2335                 2340

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Ala Cys  Cys Pro Ser Cys Arg  Cys Glu Arg Met Glu  Ala Cys Met
    2570                 2575                 2580

Leu Asn  Gly Thr Val Ile Gly  Pro Gly Lys Thr Val  Met Ile Asp
    2585                 2590                 2595
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Val Cys  Thr Thr Cys Arg Cys  Met Val Gln Val Gly  Val Ile Ser
     2600                 2605                 2610

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

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

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

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

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

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

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

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

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

Tyr Cys  Gln Gly Lys Cys Ala  Ser Lys Ala Met Tyr  Ser Ile Asp

     2750                 2755                 2760

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

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

Val Tyr  His Glu Val Leu Asn  Ala Met Glu Cys Lys  Cys Ser Pro
     2795                 2800                 2805

Arg Lys  Cys Ser Lys Ser Ser  Gly Gly Ser Gly Gly  Ser Gly Gly
     2810                 2815                 2820

Ser Gly  Gly Ser Gly Gly Ser  Gly Gly Ser Gly Gly  Ser Gly Gly
     2825                 2830                 2835

Ser Gly  Gly Ser Gly Ser Asp  Ala His Lys Ser Glu  Val Ala His
     2840                 2845                 2850

Arg Phe  Lys Asp Leu Gly Glu  Glu Asn Phe Lys Ala  Leu Val Leu
     2855                 2860                 2865

Ile Ala  Phe Ala Gln Tyr Leu  Gln Gln Cys Pro Phe  Glu Asp His
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Ile Ala  Phe Ala Gln Tyr Leu  Gln Gln Cys Pro Phe  Glu Asp His
    2870                 2875                 2880

Val Lys  Leu Val Asn Glu Val  Thr Glu Phe Ala Lys  Thr Cys Val
    2885                 2890                 2895

Ala Asp  Glu Ser Ala Glu Asn  Cys Asp Lys Ser Leu  His Thr Leu
    2900                 2905                 2910

Phe Gly  Asp Lys Leu Cys Thr  Val Ala Thr Leu Arg  Glu Thr Tyr
    2915                 2920                 2925

Gly Glu  Met Ala Asp Cys Cys  Ala Lys Gln Glu Pro  Glu Arg Asn
    2930                 2935                 2940

Glu Cys  Phe Leu Gln His Lys  Asp Asp Asn Pro Asn  Leu Pro Arg
    2945                 2950                 2955

Leu Val  Arg Pro Glu Val Asp  Val Met Cys Thr Ala  Phe His Asp
    2960                 2965                 2970

Asn Glu  Glu Thr Phe Leu Lys  Lys Tyr Leu Tyr Glu  Ile Ala Arg
    2975                 2980                 2985

Arg His  Pro Tyr Phe Tyr Ala  Pro Glu Leu Leu Phe  Phe Ala Lys
    2990                 2995                 3000

Arg Tyr  Lys Ala Ala Phe Thr  Glu Cys Cys Gln Ala  Ala Asp Lys
    3005                 3010                 3015

Ala Ala  Cys Leu Leu Pro Lys  Leu Asp Glu Leu Arg  Asp Glu Gly
    3020                 3025                 3030

Lys Ala  Ser Ser Ala Lys Gln  Arg Leu Lys Cys Ala  Ser Leu Gln
    3035                 3040                 3045

Lys Phe  Gly Glu Arg Ala Phe  Lys Ala Trp Ala Val  Ala Arg Leu
    3050                 3055                 3060

Ser Gln  Arg Phe Pro Lys Ala  Glu Phe Ala Glu Val  Ser Lys Leu
    3065                 3070                 3075

Val Thr  Asp Leu Thr Lys Val  His Thr Glu Cys Cys  His Gly Asp
    3080                 3085                 3090

Leu Leu  Glu Cys Ala Asp Asp  Arg Ala Asp Leu Ala  Lys Tyr Ile
    3095                 3100                 3105

Cys Glu  Asn Gln Asp Ser Ile  Ser Ser Lys Leu Lys  Glu Cys Cys
    3110                 3115                 3120

Glu Lys  Pro Leu Leu Glu Lys  Ser His Cys Ile Ala  Glu Val Glu
    3125                 3130                 3135

Asn Asp  Glu Met Pro Ala Asp  Leu Pro Ser Leu Ala  Ala Asp Phe
    3140                 3145                 3150

Val Glu  Ser Lys Asn Val Cys  Lys Asn Tyr Ala Glu  Ala Lys Asp
```

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Val Glu  Ser Lys Asp Val Cys  Lys Asn Tyr Ala Glu  Ala Lys Asp
    3155                 3160                 3165

Val Phe  Leu Gly Met Phe Leu  Tyr Glu Tyr Ala Arg  Arg His Pro
    3170                 3175                 3180

Asp Tyr  Ser Val Val Leu Leu  Leu Arg Leu Ala Lys  Thr Tyr Glu
    3185                 3190                 3195

Thr Thr  Leu Glu Lys Cys Cys  Ala Ala Ala Asp Pro  His Glu Cys
    3200                 3205                 3210

Tyr Ala  Lys Val Phe Asp Glu  Phe Lys Pro Leu Val  Glu Glu Pro
    3215                 3220                 3225

Gln Asn  Leu Ile Lys Gln Asn  Cys Glu Leu Phe Glu  Gln Leu Gly
    3230                 3235                 3240

Glu Tyr  Lys Phe Gln Asn Ala  Leu Leu Val Arg Tyr  Thr Lys Lys
    3245                 3250                 3255

Val Pro  Gln Val Ser Thr Pro  Thr Leu Val Glu Val  Ser Arg Asn
    3260                 3265                 3270

Leu Gly  Lys Val Gly Ser Lys  Cys Cys Lys His Pro  Glu Ala Lys
    3275                 3280                 3285

Arg Met  Pro Cys Ala Glu Asp  Tyr Leu Ser Val Val  Leu Asn Gln
    3290                 3295                 3300

Leu Cys  Val Leu His Glu Lys  Thr Pro Val Ser Asp  Arg Val Thr
    3305                 3310                 3315

Lys Cys  Cys Thr Glu Ser Leu  Val Asn Arg Arg Pro  Cys Phe Ser
    3320                 3325                 3330

Ala Leu  Glu Val Asp Glu Thr  Tyr Val Pro Lys Glu  Phe Asn Ala
    3335                 3340                 3345

Glu Thr  Phe Thr Phe His Ala  Asp Ile Cys Thr Leu  Ser Glu Lys
    3350                 3355                 3360

Glu Arg  Gln Ile Lys Lys Gln  Thr Ala Leu Val Glu  Leu Val Lys
    3365                 3370                 3375

His Lys  Pro Lys Ala Thr Lys  Glu Gln Leu Lys Ala  Val Met Asp
    3380                 3385                 3390

Asp Phe  Ala Ala Phe Val Glu  Lys Cys Cys Lys Ala  Asp Asp Lys
    3395                 3400                 3405

Glu Thr  Cys Phe Ala Glu Glu  Gly Lys Lys Leu Val  Ala Ala Ser
    3410                 3415                 3420

Gln Ala  Ala Leu Gly Leu
    3425
```

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Patentkrav

1. En modificeret Faktor VIII eller et kompleks omfattende modificeret Faktor VIII og ikke-modificeret VWF, hvor den modificerede Faktor VIII er fusioneret ved en C-terminal del af det primære translationspolypeptid fra Faktor VIII til den N-terminale del af et albumin.

2. Modificeret Faktor VIII eller et kompleks omfattende modificeret Faktor VIII og ikke-modificeret VWF ifølge krav 1, hvor

a. den modificerede Faktor VIII har en forlænget funktionel halveringstid sammenlignet med den funktionelle halveringstid af vildtype Faktor VIII eller
b. komplekset omfattende modificeret Faktor VIII og ikke-modificeret VWF har en forlænget funktionel halveringstid sammenlignet med den funktionelle halveringstid af det tilsvarende kompleks omfattende vildtype Faktor VIII og vildtype VWF.

3. Modificeret Faktor VIII eller et kompleks omfattende nævnte modificerede Faktor VIII eller et kompleks omfattende modificeret Faktor VIII og ikke-modificeret VWF ifølge krav 2, hvor den modificerede Faktor VIII har en funktionel halveringstid øget med mindst 25% sammenlignet med den funktionelle halveringstid for den tilsvarende vildtype Faktor VIII eller et kompleks omfattende nævnte modificerede Faktor VIII og ikke-modificeret VWF har en funktionel halveringstid øget med mindst 25% sammenlignet med det tilsvarende kompleks af vildtype Faktor VIII og vildtype VWF.

4. Modificeret Faktor VIII eller et kompleks omfattende modificeret Faktor VIII og ikke-modificeret VWF ifølge krav 1, hvor

a. den modificerede Faktor VIII har en forlænget antigen-halveringstid sammenlignet med antigen-halveringstiden for vildtype Faktor VIII eller
b. komplekset omfattende modificeret Faktor VIII og ikke-modificeret VWF har en forlænget antigen-halveringstid sammenlignet med antigen-halveringstiden

for det tilsvarende kompleks omfattende vildtype Faktor VIII og vildtype VWF.

5. Modificeret Faktor VIII eller et kompleks omfattende modificeret Faktor VIII og ikke-modificeret VWF ifølge krav 4, hvor den modificerede Faktor VIII har en antigen-halveringstid øget med mindst 25% sammenlignet med antigen-halveringstiden af den tilsvarende vildtype Faktor VIII eller et kompleks omfattende nævnte modificerede Faktor VIII og ikke-modificerede VWF har en antigen-halveringstid øget med mindst 25% sammenlignet med det tilsvarende kompleks af vildtype Faktor VIII og vildtype VWF.

6. Modificeret Faktor VIII eller et kompleks omfattende modificeret FVIII og ikke-modificeret VWF ifølge krav 1, hvor

a. den modificerede Faktor VIII har en øget in vivo genvinding sammenligning med in vivo genvindingen af vildtype Faktor VIII eller
b. komplekset omfattende modificeret Faktor VIII og ikke-modificeret VWF har en øget in vivo genvinding sammenlignet med in vivo genvindingen af det tilsvarende kompleks omfattende vildtype Faktor VIII og vildtype VWF.

7. Modificeret Faktor VIII eller et kompleks omfattende modificeret Faktor VIII og ikke-modificeret VWF ifølge krav 6, hvor den modificerede Faktor VIII har en in vivo genvinding øget med mindst 10% sammenlignet med in vivo genvindingen af den tilsvarende vildtype Faktor VIII eller et kompleks omfattende nævnte modificerede Faktor VIII og ikke-modificerede VWF har et in vivo genvinding øget med mindst 10% sammenlignet med det tilsvarende kompleks af vildtype Faktor VIII og vildtype VWF.

8. Modificeret Faktor VIII eller et kompleks omfattende modificeret Faktor VIII og ikke-modificeret VWF ifølge et hvilket som helst af de foregående krav, hvor den modificerede Faktor VIII har mindst 10% af den biologiske aktivitet af vildtype Faktor VIII eller komplekset omfattende det modificerede polypeptid eller et kompleks omfattende nævnte modificerede polypeptider har mindst 10% af den biologiske aktivitet af det tilsvarende kompleks af vildtype FVIII og vildtype VWF.

9. Rekombinant modificeret FVIII ifølge et hvilket som helst af de foregående krav, hvor nævnte rekombinante modificerede FVIII er udskilt fra pattedyrsceller i højere udbytte som vildtype FVIII.

10. Polynukleotid eller en gruppe af polynukleotider, kodende for en modificeret Faktor VIII ifølge et hvilket som helst af kravene 1 til 9.

11. Plasmid eller vektor omfattende et polynukleotid ifølge krav 10, hvor nævnte plasmid eller vektor er en ekspressionsvektor eller en overføringsvektor til anvendelse i human genterapi.

12. Værtscelle omfattende et polynukleotid ifølge krav 10 eller et plasmid eller en vektor ifølge krav 11.

13. Fremgangsmåde til fremstilling af en modificeret Faktor VIII omfattende:

(a) dyrkning af værtsceller ifølge krav 12 under betingelser sådan at den modificerede Faktor VIII er eksprimeret; og
(b) eventuelt genvinding af den modificerede Faktor VIII fra værtcellerne eller fra dyrkningsmediet.

14. Farmaceutisk sammensætning omfattende et polypeptid eller et kompleks omfattende nævnte modificerede Faktor VIII ifølge et hvilket som helst af kravene 1 til 9, et polynukleotid ifølge krav 10 eller et plasmid eller en vektor ifølge krav 11.

15. Anvendelse af et polypeptid eller et kompleks omfattende nævnte modificerede Faktor VII ifølge et hvilket som helst af kravene 1 til 9, et polynukleotid ifølge krav 10 eller et plasmid eller en vektor ifølge krav 11 eller en værtscelle ifølge krav 12 til fremstilling af et medikament til behandling eller forebyggelse af en blodkoagulationsforstyrrelse.

16. Anvendelse ifølge krav 15, hvor blodkoagulationsforstyrrelsen er hæmofili A.

17. Anvendelse ifølge krav 15, hvor behandlingen omfatter human genterapi.

DRAWINGS

Figure 1: Antigen and activity levels of wild-type FVIII (457) and FVIII-C-terminal (1434) albumin fusion polypeptides

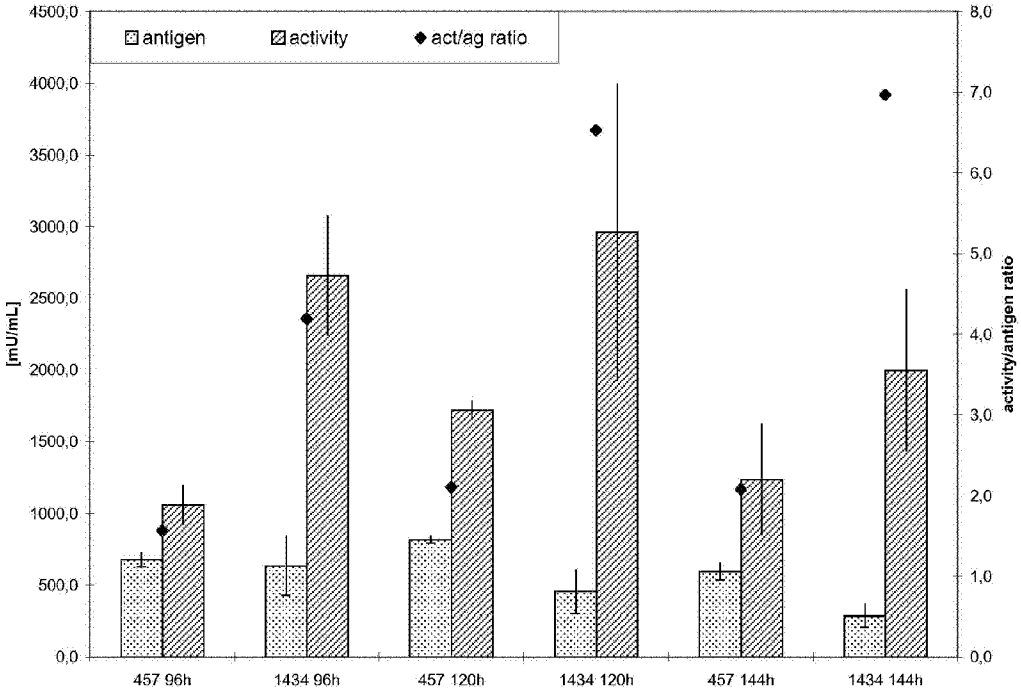


Figure 2: Comparison of human FVIII:Ag pharmacokinetics in VWF ko mice following i.v. injection of 100 U (FVIII:Ag)/kg FVIII wildtype and FVIII-FP 1656 VWF (mean; n=4/timepoint)

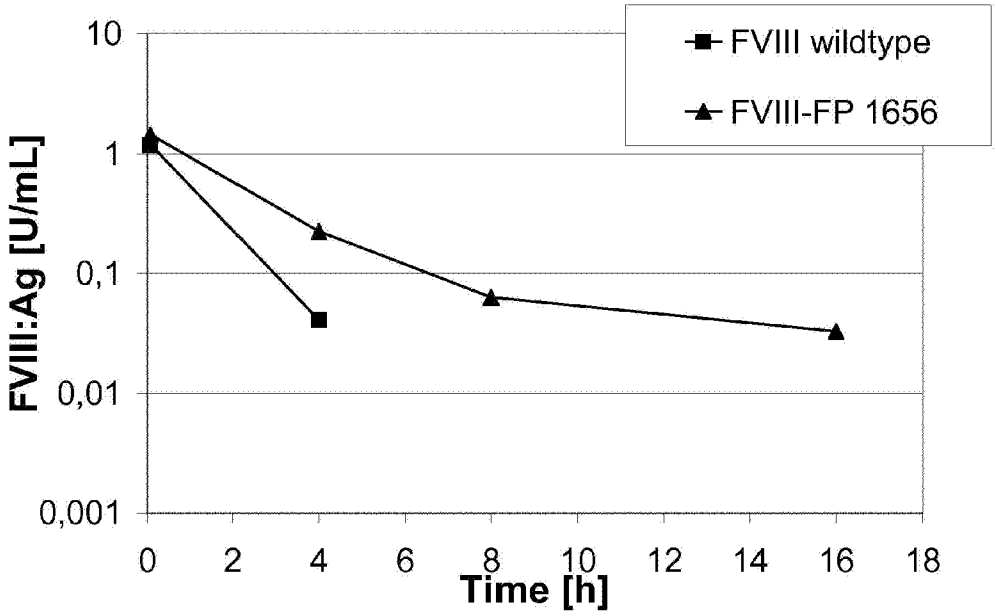


Figure 3: VWF:RCo/VWF:Ag ratios of cell culture supernatants containing wt rVWF (1570/797), rVWF-FP (1572/797) containing C-terminally linked albumin, or a mixed expression cell culture containing a mixture of wt rVWF (1570/797) and rVWF-FP (1572/797) transfected in a ratio of 5:1. Values of about 0,8 were obtained in every case that are close to 1 which is the theoretical ratio of NHP according to the unit definitions.

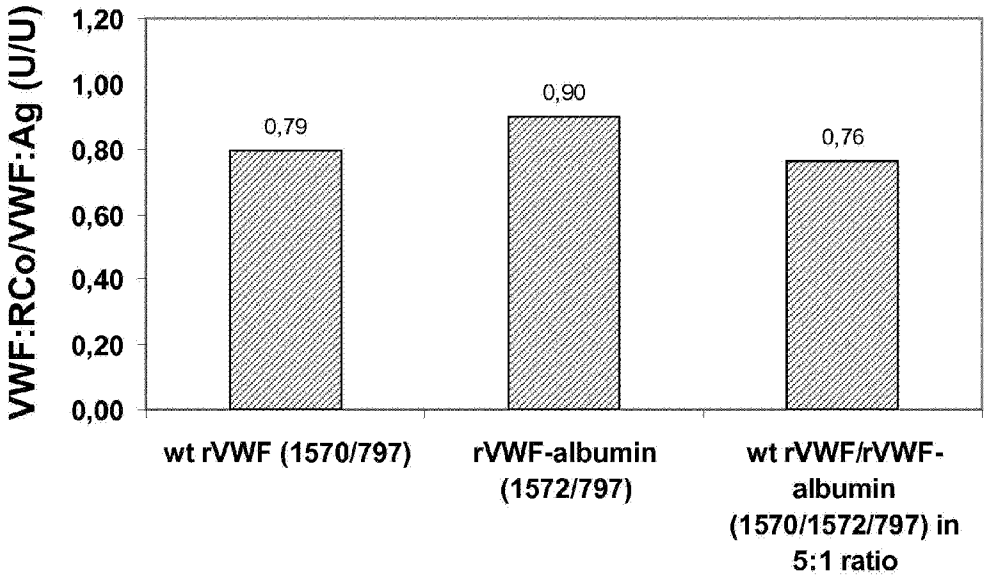
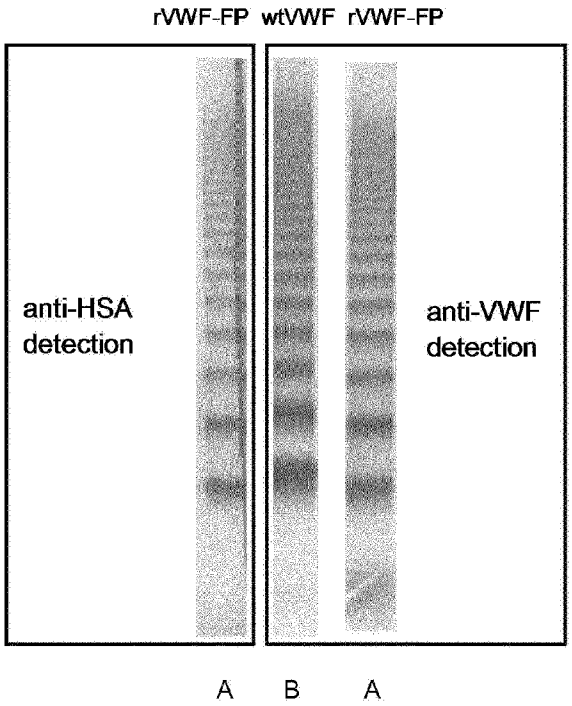


Figure 4: SDS-Agarose gel electrophoresis of wild-type rVWF (1570/797) (B) and rVWF-FP (1572/797), both expressed in HEK cells (A). Bands were detected using either antibodies to VWF or to albumin (HSA).



A = rVWF-FP (Expressed in presence of furin)

B = wt VWF (Expressed in presence of furin)

Figure 5: PK analysis of rVWF wt and rVWF-FP in rats based on VWF:Ag determination.