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(54) **Title**: FACTOR VIII, VON WILLEBRAND FACTOR OR COMPLEXES THEREOF WITH PROLONGED IN VIVO HALF-LIFE

(57) **Abstract**: The present invention relates to modified nucleic acid sequences coding for coagulation factor VIII (FVIII) and for 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.

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Factor VIII, von Willebrand factor or complexes thereof with prolonged in vivo half-life

Field of the invention:

The present invention relates to modified nucleic acid sequences coding for coagulation factor VIII (FVIII) and for 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:

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.

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.

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.

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.

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.

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.

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).

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.

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.

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).

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.

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.

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.

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.

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.

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.

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.

In plasma FVIII binds with high affinity to von 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).

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.

The in vivo half-life of human VWF in the human circulation is approximately 12 to 20 hours. In prophylactic treatment of VWD e.g. of type 3 it would also be highly desirable to find ways to prolong the functional half-life of VWF.

Another approach to enhance the functional half-life of VWF is by PEGylation (WO 2006/071801) which pegylated VWF by having an increased half-life would indirectly also enhance the half-life of FVIII present in plasma.

However the chemical conjugation of PEG or other molecules to therapeutic proteins always entails the risk, that the specific activity is reduced due to shielding of important interaction sites with other proteins, chemical conjugation adds an additional step in the manufacture of such proteins decreasing final yields and making manufacture more expensive. Also the long term effects on human health are not known as currently known PEGylated therapeutic proteins do not need to be administrated lifelong as it would be the case for a VWF to be administered in prophylaxis of von Willebrand disease or in for a FVIII to be administered in hemophilia A .

It would thus be highly desirable to obtain a long-lived VWF which is not chemically modified.

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.

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.

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.

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.

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.

A man skilled in the art would also not have considered fusing human albumin to the N- or the C-terminus of VWF. In an N-terminal fusion the albumin part would be cleaved off during propeptide processing. Or if the propeptide would be omitted the multimerization would not take place. As detailed above the C-terminus of VWF is essential for the initial dimerization and secretion as shown by Schneppenheim et al. (Schneppenheim R. et al. 1996. Defective dimerization of VWF subunits due to a Cys to Arg mutation in VWD type IID. Proc Natl Acad Sci USA 93:3581-3586; Schneppenheim R. et al. 2001. Expression and characterization of VWF dimerization defects in different types of VWD. Blood 97:2059-2066.), Baronciani et al. (Baronciani L.et al. 2000. Molecular characterization of a multiethnic group of 21 patients with VWD type 3. Thromb. Haemost 84:536-540), Enayat et al. (Enayat MS et al. 2001. Aberrant dimerization of VWF as the result of mutations in the carboxy-terminal region: identification of 3 mutations in members of 3 different families with type 2A (phenotype IID) VWD. Blood 98:674-680) and Tjernberg et al. 2006. Homozygous C2362F VWF induces intracellular retention of mutant VWF resulting in autosomal recessive severe VWD. Br J Haematol. 133:409-418). Therefore the man skilled in the art would not consider fusing a large protein like human albumin to the C- or N-terminus of VWF as he would expect that normal dimerization or multimerization of VWF would be impaired. As the higher multimers of VWF are the ones most active in primary hemostasis the man skilled in the art would have looked for other ways to prolong the functional half-life of VWF.

It was now surprisingly found that fusing heterologous polypeptides such as albumin to the C-terminal part of VWF not only permits expression and secretion of VWF chimeric proteins from mammalian cells but also results in modified VWF molecules that retain significant VWF activity and form high molecular weight multimers. In addition, such modified VWF molecules exhibit prolonged in vivo half-life and/or improved in vivo recovery.

Description of the invention

It is a desirable outcome of this invention to provide a modified FVIII or a modified VWF as well as complexes of modified FVIII with non-modified VWF, complexes of non-modified FVIII with modified VWF and also complexes of modified FVIII with modified VWF with enhanced in vivo half-life.

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

It is another desirable outcome of this invention to provide a modified FVIII or a modified VWF as well as complexes of modified FVIII with non-modified VWF, complexes of non-modified FVIII with modified VWF and also complexes of modified FVIII with modified VWF with improved in vivo recovery.

Another desirable outcome of the invention is that this modified FVIII or modified VWF as well as complexes of modified FVIII with non-modified VWF, non-modified FVIII with modified VWF and also complexes of modified FVIII with modified VWF can be expressed by mammalian cells and retain their respective biological activities.

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

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.

The modified FVIII or modified VWF as well as complexes of modified FVIII with non-modified VWF, complexes of non-modified FVIII with modified VWF and also complexes of modified FVIII with modified VWF molecules of the invention can be generated by fusing a half-life enhancing protein (HLEP) moiety to the C-terminal part of FVIII or to the C-terminal part of VWF.

HLEPs in the sense of the present invention are selected from a group consisting of members of the albumin family, which includes albumin, afamin, alpha-fetoprotein and the vitamin D binding protein, as well as portions of an immunoglobulin constant region and polypeptides capable of binding under physiological conditions to members of the albumin family as well as to portions of an immunoglobulin constant region. The most preferred HLEP is human albumin.

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

According to one embodiment, there is provided a modified VWF, or a complex comprising non-modified FVIII and modified VWF, wherein the modified VWF is created by fusing the C-terminus of the primary translation polypeptide of VWF, or a variant thereof, to the N-terminal part of a HLEP, wherein the HLEP is albumin or variants or fragments thereof, or an immunoglobulin constant region or variants or fragments thereof.

The present invention also relates to C-terminal fusions to more than one HLEP wherein the HLEP, which is fused several times, may be the same HLEP or may be a combination of different HLEPs.

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 modified VWF or the complex of modified FVIII with non-modified VWF, the complex of non-modified FVIII with modified VWF or the complex of modified FVIII with modified VWF has improved in vivo recovery compared to the in vivo recovery of the wild-type FVIII or wild-type VWF or the complex of wild-type VWF and wild-type FVIII.

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.

Another aspect of the invention are polynucleotides or combinations of polynucleotides encoding the modified FVIII and/or the modified VWF.

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.

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

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

The invention further pertains to pharmaceutical compositions comprising a modified FVIII or a modified VWF or a complex of modified FVIII with non-modified VWF or a complex of non-modified FVIII with modified VWF or a complex of modified FVIII with 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 modified VWF or a complex of modified FVIII with non-modified VWF or a complex of non-modified FVIII with modified VWF or a complex of modified FVIII with 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

The invention pertains to a complex comprising FVIII and von VWF or one of its individual polypeptidic components 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 part of a half-life enhancing polypeptide (HLEP)

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

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

a. the modified FVIII has a prolonged functional half-life compared to the functional half-life of wild-type FVIII or
b. the modified VWF has a prolonged functional half-life compared to the functional half-life of wild-type VWF or
c. 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 or

d. the complex comprising non-modified FVIII and 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 or
e. the complex of modified FVIII with 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.

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.

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

a. the modified FVIII has a prolonged antigen half-life compared to the antigen half-life of wild-type FVIII or
b. the modified VWF has a prolonged antigen half-life compared to the antigen half-life of wild-type VWF or
c. 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 or
d. the complex comprising non-modified FVIII and modified VWF has a prolonged antigen half-life compared to the antigen half-life of the corresponding complex of wild-type FVIII and wild-type VWF or
e. the complex comprising modified FVIII and modified VWF has a prolonged antigen half-life compared to the antigen half-life of the corresponding complex of wild-type FVIII and wild-type VWF.

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.

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

a. the modified FVIII has an increased in vivo recovery compared to the in vivo recovery of wild-type FVIII or
b. the modified VWF has an increased in vivo recovery compared to the in vivo recovery of wild-type VWF or
c. 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 or
d. the complex comprising non-modified FVIII and 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 or
e. the complex comprising modified FVIII and 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.

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

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
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
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.

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.

Also comprised in the present invention is a method of preparing a modified FVIII or a modified VWF 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 or to a C-terminal part of the primary translation polypeptide of the VWF as well as a method of preparing a complex comprising modified FVIII and non-modified VWF or a complex comprising non-modified FVIII and modified VWF or a complex comprising modified FVIII and modified VWF by mixing a modified FVIII prepared by the method described above with wild-type VWF or by mixing wild-type FVIII with a modified VWF prepared by the method described above or by mixing a modified FVIII and a modified VWF prepared by the method described above.

Also encompassed in the invention is the use of

a. a modified FVIII as prepared by the method described above and wild-type VWF or
b. a wild-type FVIII and a modified VWF prepared by the method described above or
c. a modified FVIII a as prepared by the method described above and a modified VWF as prepared by the method described above

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.

The “functional half-life” according to the present invention is the half-life of the biological activity of the modified FVIII or the modified VWF or a complex of modified FVIII with non-modified VWF or a complex of the non-modified FVIII with modified VWF or a complex of modified FVIII with 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 modified VWF or the complex of modified FVIII with non-modified VWF or the complex of non-modified FVIII with modified VWF or the complex of modified FVIII with modified VWF has been administered.

The phrases "fusing" or "fused" refer to the addition of amino acids to the C-terminal part of FVIII and/or to the C-terminal part of VWF. When referring herein to a “fusion to the C-terminal amino acid of FVIII” or to a “fusion to the C-terminal amino acid of VWF” 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 or exactly to the C-terminal amino acid of VWF at amino acid 2050 of wild-type mature VWF. Mature FVIII or mature VWF meaning the respective polypeptide after cleavage of the propeptide. However the invention also encompasses a “fusion to the C-terminal part of FVIII” or a “fusion to the C-terminal part of VWF” in the sense of this invention may also include a fusion to a FVIII and/or VWF molecule respectively in which one or more amino acid position up to n amino acids from the C-terminal amino acid of FVIII and/or of VWF 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 and/or VWF. Usually, n is 20, preferably 15, more preferably 10, still more preferably 5 or less (e.g. 1, 2, 3, 4 or 5).

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

In another embodiment the modified VWF has the following structure:

N – VWF – C –L1- H, [formula 2]

wherein
N is an N-terminal part of VWF,
L1 is a chemical bond or a linker sequence
H is a HLEP, and
C is a C-terminal part of VWF

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.

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.

The modified FVIII or the modified VWF or the complex of the modified FVIII with the non-modified VWF, the complex of the non-modified FVIII with the modified VWF or the complex of the modified FVIII with 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 and/or to the C-terminal part of VWF in tandem, e.g. as successive repeats.

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.

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.

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.

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.
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.

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.

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.

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.

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.

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 co-factor activity (Federici AB et al. 2004. Haematologica 89:77-85), binding of VWF to GP Ibα 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).

"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	

One or more HLEPs may 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.

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

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%).

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.

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

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.

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.

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.

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.

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.

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

The functional half-life of a modified VWF comprising a HLEP modification, can be determined by administering the respective modified VWF (and in comparison that of the non-modified VWF) 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 VWF in blood samples drawn at appropriate intervals after application. Suitable test methods are the activity tests described herein.

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 antigen of the modified or respectively wild-type VWF can be measured. Thus also encompassed by the invention are modified FVIII and/or VWF molecules having at the C-terminal part of FVIII and/or VWF a fusion to a HLEP, characterized in that the modified FVIII or the modified VWF or the modified VWF or the complex of modified FVIII with non-modified VWF, or the complex of the non-modified FVIII with modified VWF or the complex of modified FVIII with modified VWF has a prolonged half-life of the FVIII and/or VWF antigen compared to the half-life of the FVIII and/or VWF 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. The "half-life of the VWF antigen" according to the present invention is the half-life of the antigen of the VWF 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 $t_{1/2} = \ln 2 / k$, whereas k is the slope of the regression line.

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.

In another embodiment of the invention, the modified FVIII of the invention exhibits an improved in vivo recovery compared to the wild-type FVIII and the modified VWF of the invention exhibits an improved in vivo recovery compared to the wild-type VWF. 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, or in models of VWD, like VWF knockout mice in which one would expect an increased percentage of the modified FVIII or VWF 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.

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.

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).

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%).

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.

It is another objective of the present invention to provide long-lived VWF molecules, which after proteolytic processing in vivo do have functional properties comparable to that of an unmodified VWF. This can be achieved by maintaining or inserting certain cleavage sites in the modified VWF (see infra) leading to a proteolytic cleavage for example when in contact with activated coagulation factors, which separates the VWF from the HLEP. Accordingly, in one embodiment, the functional half-life of the proteolytically processed modified VWF 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%).

Another preferred embodiment of the invention is a coexpression of wild-type VWF and a modified VWF according to the invention resulting in VWF multimers comprising non-modified as well as modified VWF monomers.

Linker sequences

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.

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

In another embodiment of the invention the peptidic linker between the 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.

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.

The cleavable linker preferably comprises a sequence derived from

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

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.

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.

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.

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

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

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)

A "half-life enhancing polypeptide" as used herein is selected from the group consisting of albumin, a member of the albumin-family, the constant region of immunoglobulin G and fragments thereof region and polypeptides capable of binding under physiological conditions to albumin, to members of the albumin family as well as to portions of an immunoglobulin constant region. It may be a full-length half-life-enhancing protein described herein (e.g. albumin, a member of the albumin-family or the constant region of immunoglobulin G) or one or more fragments thereof that are capable of stabilizing or prolonging the therapeutic activity or the biological activity of the coagulation factor. Such fragments may be of 10 or more amino acids in length or may include at least about 15, at least about 20, at least about 25, at least about 30, at least about 50, at least about 100, or more contiguous amino acids from the HLEP sequence or may include part or all of specific domains of the respective HLEP, as long as the HLEP fragment provides a functional half-life extension of at least 25% compared to a wild-type FVIII or wild-type VWF.

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.

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

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).

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.

In particular, the proposed FVIII fusion and/or VWF 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.

The albumin portion of the proposed FVIII fusion and/or VWF fusion constructs of the invention may comprise at least one subdomain or domain of HA or conservative modifications thereof.

Afamin, alpha-fetoprotein and vitamin D binding protein as HLEPs

Besides albumin, alpha-fetoprotein, another member of the albumin family, has been claimed to enhance the half-life of an attached therapeutic polypeptide in vivo (WO 2005/024044). The albumin family of proteins, evolutionarily related serum transport proteins, consists of albumin, alpha-fetoprotein (AFP; Beattie & Dugaiczyk 1982. Gene 20:415-422), afamin (AFM; Lichenstein et al. 1994. J. Biol. Chem. 269:18149-18154) and vitamin D binding protein (DBP; Cooke & David 1985. J. Clin. Invest. 76:2420-2424). Their genes represent a multigene cluster with structural and functional similarities mapping to the same chromosomal region in humans, mice and rat. The structural similarity of the albumin family members suggest their usability as HLEPs. It is therefore another object of the invention to use such albumin family members, fragments and variants thereof as HLEPs. The term "variants" includes insertions, deletions and substitutions, either conservative or non-conservative as long as the desired function is still present.
Albumin family members may comprise the full length of the respective protein AFP, AFM and DBP, or may include one or more fragments thereof that are capable of stabilizing or prolonging the therapeutic activity. Such fragments may be of 10 or more amino acids in length or may include about 15, 20, 25, 30, 50, or more contiguous amino acids of the respective protein sequence or may include part or all of specific domains of the respective protein, as long as the HLEP fragments provide a half-life extension of at least 25%. Albumin family members of the insertion proteins of the invention may include naturally occurring polymorphic variants of AFP, AFM and DBP.

Immunoglobulins as HLEPs

Immunoglobulin G (IgG) constant regions (Fc) are known in the art to increase the half-life of therapeutic proteins (Dumont JA et al. 2006. BioDrugs 20:151-160). The IgG constant region of the heavy chain consists of 3 domains (CH1 – CH3) and a hinge region. The immunoglobulin sequence may be derived from any mammal, or from subclasses IgG1, IgG2, IgG3 or IgG4, respectively. IgG and IgG fragments without an antigen-binding domain may also be used as HLEPs. The therapeutic polypeptide portion is connected to the IgG or the IgG fragments preferably via the hinge region of the antibody or a peptidic linker, which may even be cleavable. Several patents and patent applications describe the fusion of therapeutic proteins to immunoglobulin constant regions to enhance the therapeutic protein´s in vivo half-lifes. US 2004/0087778 and WO 2005/001025 describe fusion proteins of Fc domains or at least portions of immunoglobulin constant regions with biologically active peptides that increase the half-life of the peptide, which otherwise would be quickly eliminated in vivo. Fc-IFN-ß fusion proteins were described that achieved enhanced biological activity, prolonged circulating half-life and greater solubility (WO 2006/000448). Fc-EPO proteins with a prolonged serum half-life and increased in vivo potency were disclosed (WO 2005/063808) as well as Fc fusions with G-CSF (WO 2003/076567), glucagon-like peptide-1 (WO 2005/000892), clotting factors (WO 2004/101740) and interleukin-10 (US 6,403,077), all with half-life enhancing properties.

Polynucleotides

The invention further relates to a polynucleotide encoding a modified coagulation factor, preferably a modified FVIII and/or modified VWF 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.

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.

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.

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/or the modified VWF, and a second polynucleotide may encode the C-terminal part of the modified FVIII and/or the modified VWF.

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.

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 VIII, 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.

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.

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.

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.

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:

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

It is preferred to purify the modified FVIII and/or the modified VWF 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 and/or the modified VWF of the invention is substantially free of other, non-related polypeptides.

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

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 and/or the modified VWF or the modified VWF or the complex of modified FVIII with non-modified VWF, or the complex of the non-modified FVIII with modified VWF or the complex of modified FVIII with 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

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.

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 use are monkey COS-cells, mouse L-cells, mouse C127-cells, hamster BHK-21 cells, human embryonic kidney 293 cells, and hamster CHO-cells.

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.

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.

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.

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.

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

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

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

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.

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

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.

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

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.

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.

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.

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 or the combination of non-modified FVIII with modified VWF or the combination of modified FVIII with modified VWF.

Figures

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

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. 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.

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.

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.

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.

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

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.

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

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.

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

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.
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.
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

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.

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

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 $t_{1/2} = \ln 2 / k$, 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

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.

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.

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.

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

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

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.

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.

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.

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

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.

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

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-live was calculated using the time points of the beta phase of elimination according to the formula $t_{1/2} = \ln 2 / k$, whereas k is the slope of the regression line.

The result is depicted in Figure 5 (n=2/timepoint; mean). The terminal half-lifes 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.

Comprises/comprising and grammatical variations thereof when used in this specification are to be taken to specify the presence of stated features, integers, steps or components or groups thereof, but do not preclude the presence or addition of one or more other features, integers, steps, components or groups thereof.

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CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A modified VWF, or a complex comprising non-modified FVIII and modified VWF, wherein the modified VWF is created by fusing the C-terminus of the primary translation polypeptide of VWF, or a variant thereof, to the N-terminal part of a HLEP, wherein the HLEP is albumin or variants or fragments thereof, or an immunoglobulin constant region or variants or fragments thereof.

2. A modified VWF, or a complex comprising non-modified FVIII and modified VWF, according to claim 1 wherein the variant of the primary translation polypeptide of VWF is a deletant.

3. The modified VWF, or the complex comprising non-modified FVIII and modified VWF, according to claim 2, wherein said deletant comprises N-terminal, C-terminal and/or internal deletions of the primary translation polypeptide of VWF.

4. The modified VWF, or the complex comprising non-modified FVIII and modified VWF, according to any one of the previous claims, wherein the HLEP is fused to an amino acid of VWF located at a distance from the C-terminal amino acid of up to 5% of the total length of the VWF primary translation polypeptide, based on the total number of amino acids in the VWF primary translation polypeptide.

5. The modified polypeptide or complex according to any one of the previous claims, wherein 1 to 20 amino acids at the C-terminus of the VWF primary translation polypeptide have been deleted, and wherein the resulting C-terminal amino acid of the VWF polypeptide is fused to the N-terminal amino acid of the HLEP.

6. The modified polypeptide or complex according to any one of the previous claims, wherein the C-terminal amino acid of the VWF primary translation polypeptide has been deleted, and wherein the resulting C-terminal amino acid of the VWF polypeptide is fused to the N-terminal amino acid of the HLEP.

7. The modified VWF, or the complex comprising non-modified FVIII and modified VWF, according to any one of the previous claims, wherein

a. the modified VWF has a prolonged functional half-life compared to the functional half-life of wild-type VWF, or

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b. the complex comprising non-modified FVIII and 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.

8. The modified VWF, or the complex comprising non-modified FVIII and modified VWF, according to according to any one of the previous claims, wherein

a. the modified VWF has a functional half-life increased by at least 25% as compared to the functional half-life of the corresponding wild-type polypeptide, or

b. the complex comprising said modified VWF has a functional half-life increased by at least 25% as compared to the corresponding complex of wild-type FVIII and wild-type VWF.

9. The modified VWF or the complex comprising non-modified FVIII and modified VWF according to according to any one of the previous claims, wherein

a. the modified VWF has a prolonged antigen half-life compared to the antigen half-life of wild-type VWF, or

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

10. The modified VWF, or the complex comprising non-modified FVIII and modified VWF, according to claim 9, wherein

a. the modified VWF has an antigen half-life increased by at least 25% as compared to the antigen half-life of the corresponding wild-type VWF, or

b. the complex comprising said modified VWF has an antigen half-life increased by at least 25% as compared to the corresponding complex of wild-type FVIII and wild-type VWF.

11. The modified VWF or the complex comprising non-modified FVIII and modified VWF according to any one of the previous claims, wherein

a. the modified VWF has an increased in vivo recovery compared to the in vivo recovery of wild-type VWF, or

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b. the complex comprising non-modified FVIII and 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.

12. The modified VWF, or the complex comprising non-modified FVIII and modified VWF, according to claim 11, wherein

a. the modified VWF has an in vivo recovery increased by at least 10% as compared to the in vivo recovery of the corresponding wild-type VWF, or

b. the complex comprising said modified VWF has an in vivo recovery increased by at least 10% as compared to the corresponding complex of wild-type FVIII and wild-type VWF.

13. The modified VWF, or the complex comprising non-modified FVIII and modified VWF, according to any one of the previous claims, wherein

a. the modified VWF has at least 10% of the biological activity of wild-type VWF, or

b. the complex comprising the modified VWF has at least 10% of the biological activity of the corresponding complex of wild-type FVIII and wild-type VWF.

14. An isolated polynucleotide, or a group of isolated polynucleotides, encoding a modified VWF or a complex comprising said modified VWF, according to any one of claims 1 to 13.

15. A plasmid or vector comprising an isolated polynucleotide according to claim 14, or a group of plasmids or vectors, said group comprising the group of isolated polynucleotides according to claim 14.

16. A host cell comprising an isolated polynucleotide or a group of polynucleotides according to claim 14, or a plasmid or vector or a group of plasmids or vectors according to claim 15.

17. A method of producing a modified VWF, comprising:

(a) culturing host cells according to claim 16 under conditions such that the modified VWF is expressed; and

(b) optionally recovering the modified VWF from the host cells or from the culture medium.

18. A pharmaceutical composition comprising a modified VWF, or a complex comprising said modified VWF, according to any one of claims 1 to 13, a polynucleotide or group of polynucleotides according to claim 14, or a plasmid or vector or a group of plasmids or vectors according to claim 15.

19. The use of a modified VWF, or a complex comprising said modified VWF, according to any one of claims 1 to 13, a polynucleotide or group of polynucleotides according to claim 14, or a plasmid or vector or group of plasmids or vectors according to claim 15, or a host cell according to claim 16, in the manufacture of a medicament for the treatment or prevention of a blood coagulation disorder.

20. A method of treating or preventing a blood coagulation disorder, wherein a subject in need is administered an effective amount of a modified VWF or a complex comprising said modified VWF, according to any one of claims 1 to 13, a polynucleotide or group of polynucleotides according to claim 14, or a plasmid or vector or group of plasmids or vectors according to claim 15, or a host cell according to claim 16, or medicament made according to claim 19.

21. The use according to claim 19 or method of claim 20, wherein the blood coagulation disorder is hemophilia A.

22. The use according to claim 19 or method of claim 20, wherein the blood coagulation disorder is von Willebrand disease.

23. The use according to any one of claims 19, 21 or 22, or method of any one of claims 20 to 22, wherein the treatment comprises human gene therapy.

24. A method of preparing a modified VWF having increased functional half-life, comprising fusing the N-terminal part of a half-life-enhancing polypeptide to a C-terminus of the primary translation polypeptide of VWF, or a variant thereof.

25. The method of claim 24, wherein the variant of the primary translation polypeptide of VWF is a deletant.

26. A method of preparing a complex comprising non-modified FVIII and modified VWF by mixing wild-type FVIII with a modified VWF prepared by the method of claim 24 or 25.

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27. A modified VWF, or a complex comprising non-modified FVIII and modified VWF, according to any one of claims 1 to 13, an isolated polynucleotide, or a group of isolated polynucleotides according to claim 14, a plasmid or vector according to claim 15, a host cell according to claim 16, a method according to any one of claims 17, or 20 to 26, a pharmaceutical composition according to claim 18, a use according to any one of claims 19 or 21 to 23, substantially as hereinbefore described.

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Figure 1: Antigen and activity levels of wild-type FVIII (457) and FVIII-C-terminal (1434) albumin fusion polypeptides

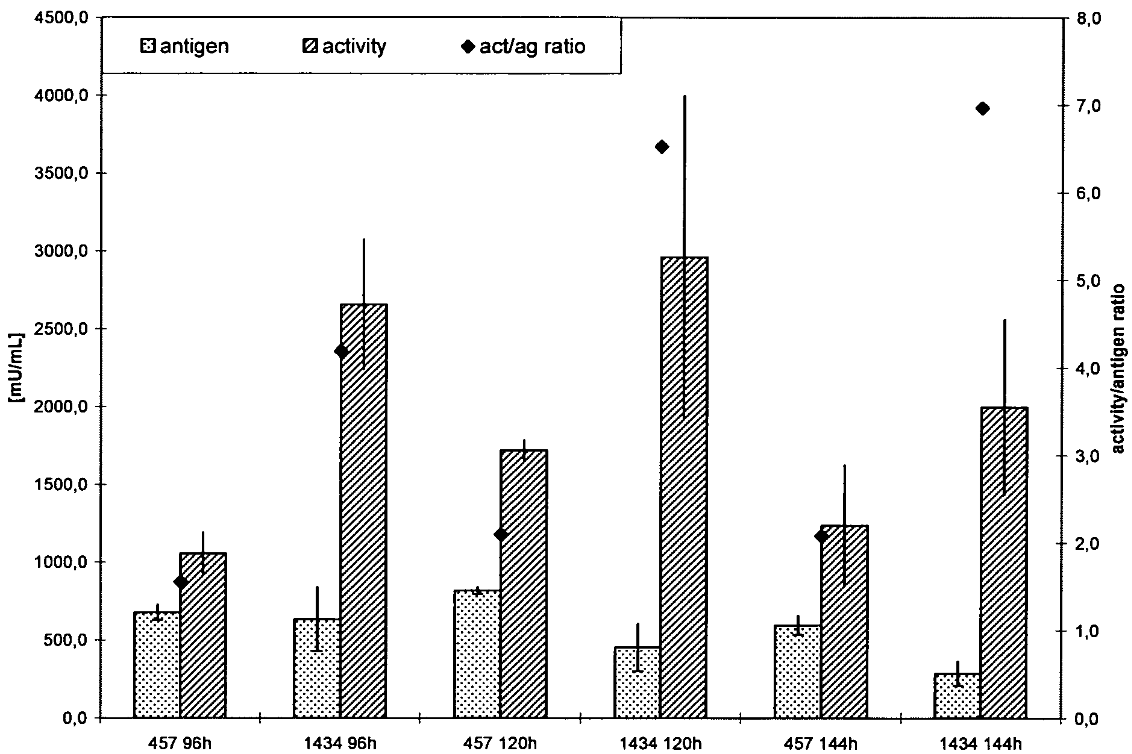


Figure 2: Comparison of human FVIII:Ag pharmacokinetics in VWF ko mice following i.v. injection of 100 U (FVIII:Ag)/kg FVIII wildtype and FVIII-FP 1656 VWF (mean; n=4/timepoint)

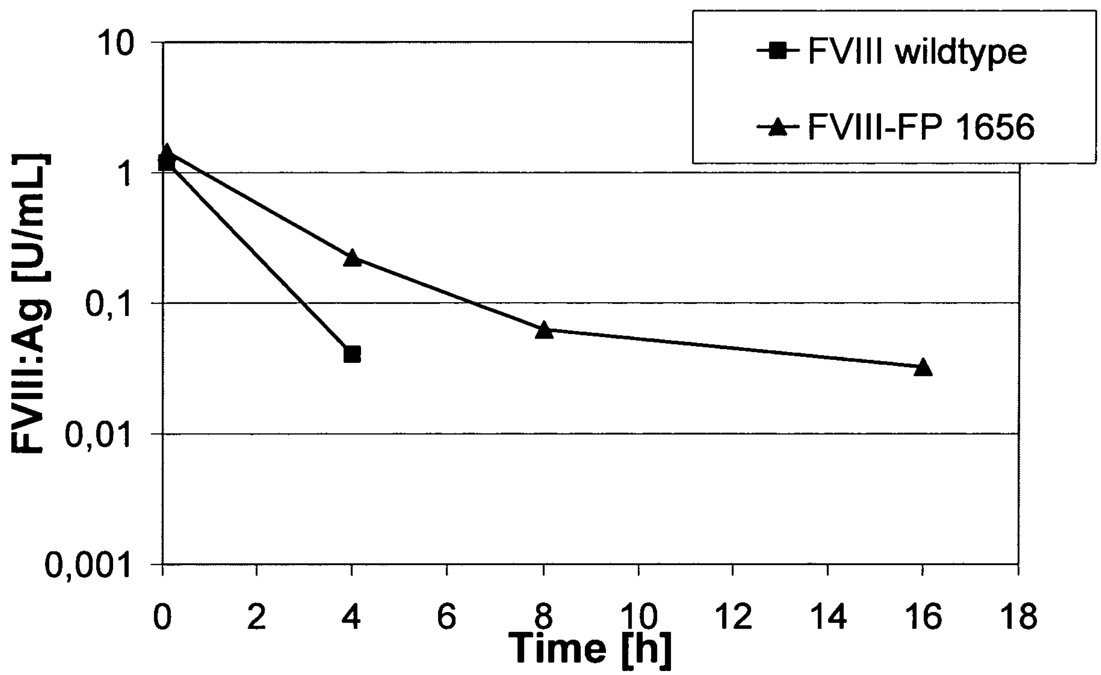


Figure 3: VWF:RCo/VWF:Ag ratios of cell culture supernatants containing wt rVWF (1570/797), rVWF-FP (1572/797) containing C-terminally linked albumin, or a mixed expression cell culture containing a mixture of wt rVWF (1570/797) and rVWF-FP (1572/797) transfected in a ratio of 5:1. Values of about 0,8 were obtained in every case that are close to 1 which is the theoretical ratio of NHP according to the unit definitions.

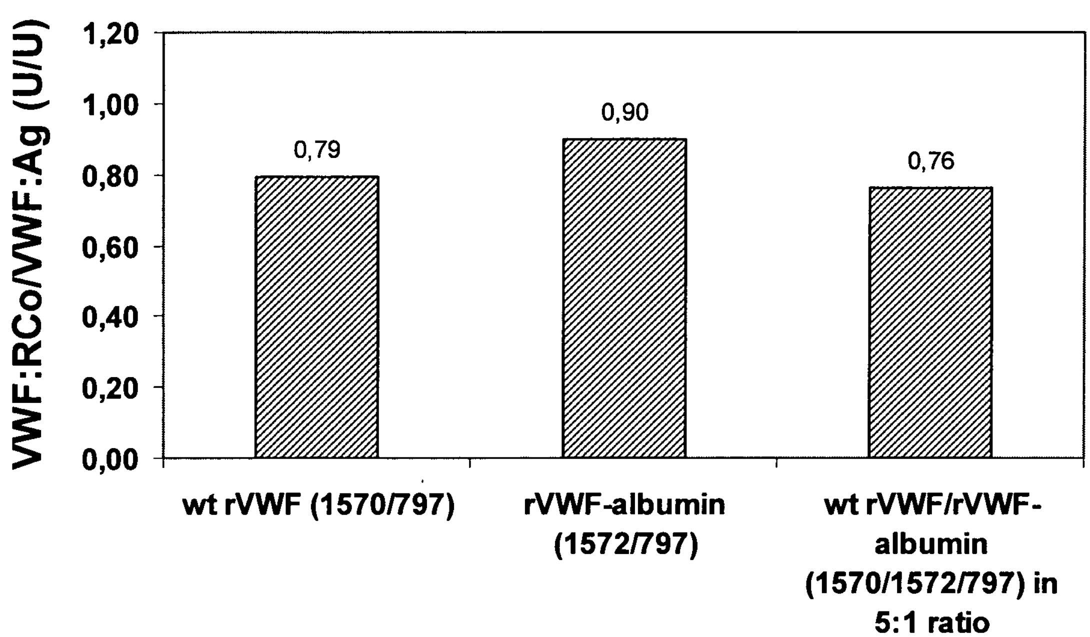
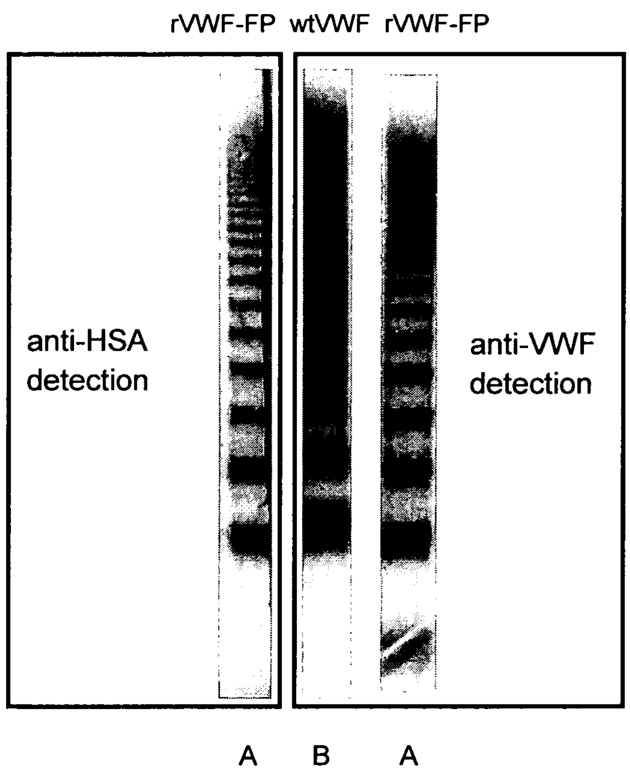


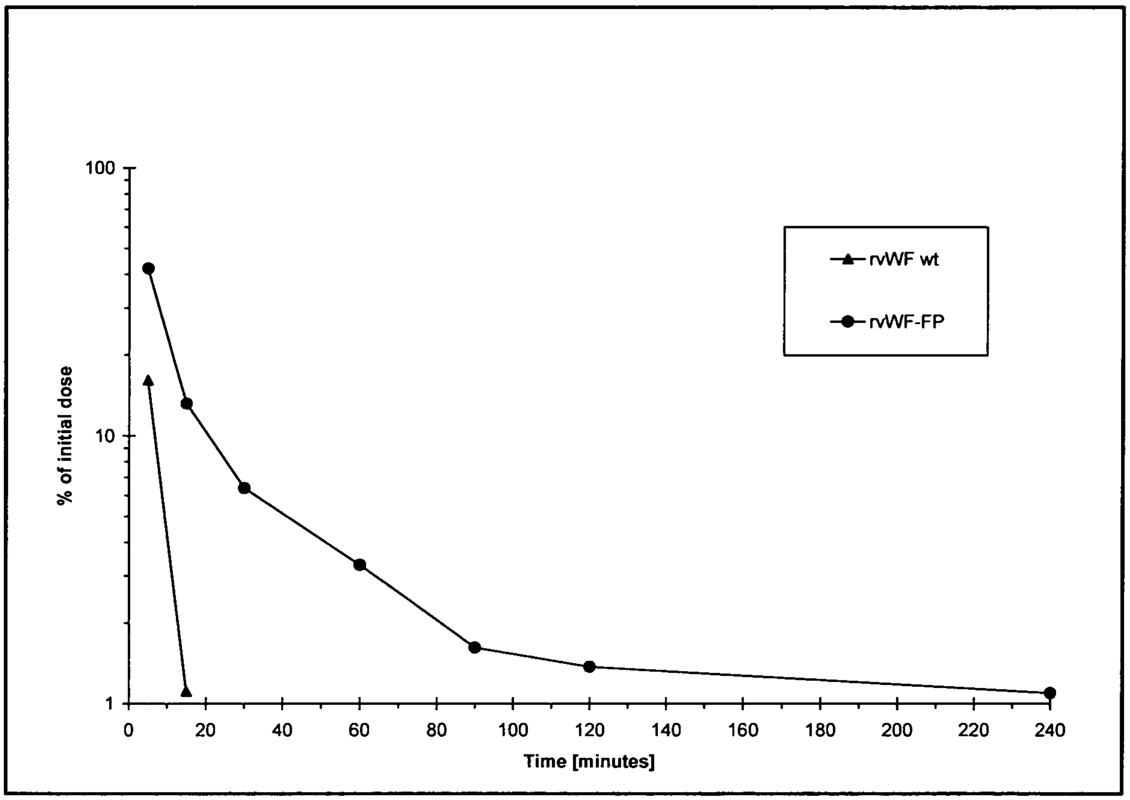
Figure 4: SDS-Agarose gel electrophoresis of wild-type rVWF (1570/797) (B) and rVWF-FP (1572/797), both expressed in HEK cells (A). Bands were detected using either antibodies to VWF or to albumin (HSA).



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.



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Leu Thr Arg Tyr Leu Arg Ile His Pro Gln Ser Trp Val His Gln

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1415 1420 1425

Tyr Thr Gly Asp Ala His Lys Ser Glu Val Ala His Arg Phe Lys
1430 1435 1440

Asp Leu Gly Glu Glu Asn Phe Lys Ala Leu Val Leu Ile Ala Phe
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Ala Gln Tyr Leu Gln Gln Cys Pro Phe Glu Asp His Val Lys Leu
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Val Asn Glu Val Thr Glu Phe Ala Lys Thr Cys Val Ala Asp Glu
1475 1480 1485

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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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
1550 1555 1560

Thr Phe Leu Lys Lys Tyr Leu Tyr Glu Ile Ala Arg Arg His Pro
1565 1570 1575

Tyr Phe Tyr Ala Pro Glu Leu Leu Phe Phe Ala Lys Arg Tyr Lys
1580 1585 1590

Ala Ala Phe Thr Glu Cys Cys Gln Ala Ala Asp Lys Ala Ala Cys
1595 1600 1605

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

Ser Ala Lys Gln Arg Leu Lys Cys Ala Ser Leu Gln Lys Phe Gly
1625 1630 1635

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

1655 1660 1665

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Cys Ala Asp Asp Arg Ala Asp Leu Ala Lys Tyr Ile Cys Glu Asn
1685 1690 1695

Gln Asp Ser Ile Ser Ser Lys Leu Lys Glu Cys Cys Glu Lys Pro
1700 1705 1710

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1715 1720 1725

Met Pro Ala Asp Leu Pro Ser Leu Ala Ala Asp Phe Val Glu Ser
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Lys Asp Val Cys Lys Asn Tyr Ala Glu Ala Lys Asp Val Phe Leu
1745 1750 1755

Gly Met Phe Leu Tyr Glu Tyr Ala Arg Arg His Pro Asp Tyr Ser
1760 1765 1770

Val Val Leu Leu Leu Arg Leu Ala Lys Thr Tyr Glu Thr Thr Leu
1775 1780 1785

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
1850 1855 1860

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
1880 1885 1890

Leu His Glu Lys Thr Pro Val Ser Asp Arg Val Thr Lys Cys Cys
1895 1900 1905

Thr Glu Ser Leu Val Asn Arg Arg Pro Cys Phe Ser Ala Leu Glu

1910 1915 1920

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

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

Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser Tyr Leu Ser
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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
180 185 190

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

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

325 330 335

Met Lys Asn Asn Glu Glu Ala Glu Asp Tyr Asp Asp Asp Leu Thr Asp
340 345 350

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Ile Gln Ile Arg Ser Val Ala Lys Lys His Pro Lys Thr Trp Val His
370 375 380

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

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

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

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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
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<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

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

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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<211> 3429
<212> PRT
<213> Artificial

<220>
<223> Amino acid sequence of human VWF albumin fusion preproprotein

<400> 25

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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

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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
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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

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
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Leu Gln Pro Thr Leu Thr Asn Pro Gly Glu Cys Arg Pro Asn Phe
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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
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Cys Asp Glu Tyr Glu Cys Ala Cys Asn Cys Val Asn Ser Thr Val
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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
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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 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
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 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