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(VON WILLEBRAND FACTOR VARIANTS HAVING IMPROVED FACTOR VIII BINDING AFFINITY)

The present invention relates to a polypeptide comprising a modified von Willebrand Factor (VWF) having a higher Factor VIII binding affinity than non-modified VWF, its pharmaceutical use and method of its preparation.
Von Willebrand Factor variants having improved Factor VIII binding affinity

FIELD OF THE INVENTION

The present invention relates to a polypeptide comprising a modified von Willebrand Factor which exhibits improved binding affinity to Factor VIII. The invention further relates to a complex comprising the polypeptide and FVIII, to a polynucleotide encoding the polypeptide of the invention and a method of producing the polypeptide. Furthermore, the invention concerns the therapeutic or prophylactic use of the polypeptide or complex of the invention for treating bleeding disorders.

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.

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 02/060951 A2), 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
(WO 2006/071801) which pegylated VWF by having an increased half-life would indirectly also enhance the half-life of FVIII present in plasma. Also fusion proteins of FVIII have been described (WO 2004/101740, WO2008/077616 and WO 2009/156137).

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 endoplasmic reticulum a C-terminal disulfide bridge is formed between two monomers of VWF. During further transport through the secretory pathway 12 N-linked and 10 O-linked carbohydrate side chains are added. 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 WVF, 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).

There is a need for VWF molecules having improved affinity to FVIII in order to stabilize FVIII. It was surprisingly found that mutations in the D’ domain of VWF can increase the affinity of VWF to FVIII. This allows providing FVIIIa/WF complexes having a high affinity which are advantageous in therapy and prophylaxis of bleeding disorder.

**SUMMARY OF THE INVENTION**

In a first aspect the present invention relates to a polypeptide comprising a modified von Willebrand Factor (VWF), wherein the amino acid sequence of said modified VWF has at least one mutation within the D’ domain relative to the amino acid sequence of the D’ domain of wild type VWF as shown in SEQ ID NO:31,
and wherein the binding affinity of said polypeptide comprising a modified VWF to Factor VIII (FVIII) is higher than that of a reference polypeptide, wherein the amino acid sequence of said reference polypeptide is identical to the amino acid sequence of said polypeptide comprising a modified VWF except that the amino acid sequence of the D' domain of the reference polypeptide consists of SEQ ID NO:31.

According to a preferred embodiment of the first aspect, the Factor VIII binding affinity of the polypeptide exceeds that of the reference polypeptide by at least 10 percent.

In another preferred embodiment, the affinity constant $K_A$ for binding of the polypeptide to wild type Factor VIII is at least $3 \times 10^{-10}$ M$^{-1}$.

In one aspect, the invention relates to a polypeptide comprising a modified von Willebrand Factor (VWF), wherein the amino acid sequence of said modified VWF has at least one substitution within the D' domain relative to the amino acid sequence of the D' domain of wild type VWF as shown in SEQ ID NO:31, wherein said substitution replaces a negatively charged amino acid present in the D' domain of wild type VWF as shown in SEQ ID NO:31 with a neutral amino acid or with a positively charged amino acid.

In another aspect, the invention relates to a polypeptide comprising a modified von Willebrand Factor (VWF), wherein the amino acid sequence of said modified VWF has at least one substitution within the D' domain relative to the amino acid sequence of the D' domain of wild type VWF as shown in SEQ ID NO:31, wherein said substitution replaces a neutral amino acid present in the D' domain of wild type VWF as shown in SEQ ID NO:31 with a positively charged amino acid.

It is preferred that the mutation within the D' domain includes an amino acid substitution at one of positions 779, 781, 787, 789, 793, 794, 796, 798, 802, 818, 819, 825, 835, 838 and 853 of the VWF amino acid sequence as shown in SEQ ID NO:2.
It is further preferred that the mutation within the D' domain includes an amino acid substitution at one of positions 779, 781, 789, 793, 794, 802, 818, 819, 835, 838 and 853 of the VWF amino acid sequence as shown in SEQ ID NO:2. For example, the amino acid substitution within the D' domain may be selected from the group consisting of Asp779Asn, Leu781Pro, Glu787Gln, Thr789Ala, Gln793Arg, Asn794Lys, Asp796Ala, Glu798Gln, Met802Arg, Met802Lys, Glu818Ala, Glu818Lys, Asn819Lys, Glu825Lys, Glu835Gln, Pro838Lys, and Asp853Asn, wherein the numbering refers to SEQ ID NO:2.

In another embodiment, the polypeptide comprises an amino acid sequence as shown in SEQ ID NO:33 or SEQ ID NO:34, with the proviso that the D' domain of the modified VWF contains at least one substitution relative to SEQ ID NO:31. For example, the polypeptide may comprise an amino acid sequence as shown in SEQ ID NO:35, with the proviso that the D' domain of the modified VWF contains at least one substitution relative to SEQ ID NO:31.

In another embodiment, the polypeptide of the present invention comprises an amino acid sequence as shown in SEQ ID NO:2 with the proviso that the D' domain of the modified VWF contains at least one amino acid substitution which increased FVIII binding by improving electrostatic attraction between the VWF polypeptide of the invention and FVIII, characterized in that acidic residues of the VWF D' domain are replaced by neutral or basic amino acids or neutral residues are replaced by basic amino acids.

In another preferred embodiment, the polypeptide of the present invention further comprises a half-life enhancing protein (HLEP). Preferably, the HLEP is an albumin. The N-terminus of the albumin may be fused to the C-terminus of the VWF amino acid sequence.

A second aspect of the present invention is a complex comprising a Factor VIII molecule and a polypeptide of the present invention. Preferably, the complex has a dissociation constant $K_D$ of 0.2 nmol/L or less. More preferably, the Factor VIII in the complex is the polypeptide of SEQ ID NO:37.
Yet another aspect of the present invention is the polypeptide of the present invention or the complex of the present invention for use in the treatment or prophylaxis of a bleeding disorder, e.g. of von Willebrand's disease (VWD) or hemophilia.

Yet another aspect of the present invention is a pharmaceutical composition comprising the polypeptide of the present invention or the complex of the present invention.

In another aspect, the invention relates to a method of treating a bleeding disorder, comprising administering to a patient in need thereof, a pharmaceutically effective amount of the polypeptide of the present invention or of the complex of the present invention. Preferably, the bleeding disorder is VWD or hemophilia A.

In yet another aspect the invention relates to a polynucleotide encoding the polypeptide of the present invention.

In another aspect, the invention pertains to a plasmid or vector comprising the polynucleotide of the present invention. The plasmid or vector is preferably an expression plasmid or expression vector.

In another aspect, the invention concerns a host cell comprising the polynucleotide or the plasmid of the present invention.

The invention further includes a method of producing a polypeptide comprising a modified VWF, comprising

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

Yet another aspect of this invention is a method of increasing the Factor VIII binding affinity of VWF, comprising introducing a mutation into the D' domain of the VWF amino acid sequence, which is not present in the amino acid sequence of the D' domain of wild type VWF as shown in SEQ ID NO:31.
In another aspect, the invention relates to the use of a modified VWF having a higher affinity to FVIII than non-modified VWF for increasing the half-life of FVIII. The modified VWF is preferably a polypeptide of the invention as defined herein, or a modified VWF as defined herein. More preferably, the modified VWF is a fusion protein, most preferably an albumin fusion protein.

A further aspect of the invention is a method of preparing a complex comprising Factor VIII and VWF, said method comprising mixing a Factor VIII molecule with the polypeptide of the present invention or its half-life extended version.

**Detailed description**

The polypeptide of the present invention comprises a modified von Willebrand Factor.

**VWF**

The term "von Willebrand Factor" or "VWF", as used herein, refers to any polypeptide having the biological activity of wild type VWF. The gene encoding wild type VWF is transcribed into a 9 kb mRNA which is translated into a pre-propolypeptide of 2813 amino acids with an estimated molecular weight of 310,000 Da. The pre-propolypeptide contains a 22 amino acids signal peptide, a 741 amino acid pro-polypeptide and the mature subunit. Cleavage of the 741 amino acids propolypeptide from the N-terminus results in mature VWF consisting of 2050 amino acids. The amino acid sequence of the VWF pre-propolypeptide is shown in SEQ ID NO:2. Unless indicated otherwise, the amino acid numbering of VWF residues in this application refers to SEQ ID NO:2, even if the VWF molecule does not need to comprise all residues of SEQ ID NO:2. The amino acid sequence of mature VWF is shown in SEQ ID NO:32. The term "VWF" as used hererein refers to the mature form of VWF unless indicated otherwise.

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

\[D1-D2-D'-D3-A1 -A2-A3-D4-B1 -B2-B3-C1 -C2-CK\]
The D1 and D2 domain represent the propeptide which is cleaved off to yield the mature VWF. The D' domain encompasses amino acids 764 to 865 of SEQ ID NO:2. The amino acid sequence of the D' domain of wild type VWF is shown in SEQ ID NO:31. The carboxyterminal 90 residues comprise the "CK" domain that is homologous to the "cystine knot" superfamily of protein. These family members have a tendency to dimerise through disulfide bonds.

Preferably, wild type VWF comprises the amino acid sequence of mature VWF as shown in SEQ ID NO:32. Also encompassed are additions, insertions, 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).

Factor VIII

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.

Preferably FVIII comprises the full length sequence of FVIII as shown in SEQ ID NO:36. Also encompassed are additions, insertions, substitutions, N-terminal, C-terminal or internal deletions of FVIII as long as the biological activity of FVIII is retained. The biological activity is retained in the sense of the invention if the FVIII with modifications retains at least 10%, preferably at least 25%, more preferably at least 50%, most preferably at least 75% of the biological activity of wild-type FVIII. The biological activity of FVIII can be determined by the artisan as described below.

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 FIIa in Bloom ed. The Hemophilias. NY Churchhill 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 amino acid sequence of the mature wild-type form of human blood coagulation FVIII is shown in SEQ ID NO:36. 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:36 does not exclude that in the modified homologue one or more amino acids at positions 1 through 2332 of SEQ ID NO:36 are missing. "FVIN" and/or "VWF" within the above definition also include natural allelic variations that may exist and occur from one individual to another. "FVIN" 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 1.

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<table>
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<tr>
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<tbody>
<tr>
<td>(1)</td>
<td>Alanine</td>
<td>Glycine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(2)</td>
<td>Aspartic acid</td>
<td>Glutamic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(3)</td>
<td>Asparagine</td>
<td>Glutamine</td>
<td>Serine</td>
<td>Threonine</td>
</tr>
<tr>
<td>(4)</td>
<td>Arginine</td>
<td>Histidine</td>
<td>Lysine</td>
<td></td>
</tr>
<tr>
<td>(5)</td>
<td>Isoleucine</td>
<td>Leucine</td>
<td>Methionine</td>
<td>Valine</td>
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<tr>
<td>(6)</td>
<td>Phenylalanine</td>
<td>Tyrosine</td>
<td>Tryptophane</td>
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</table>

Table 1:

Modified VWF

The modified VWF of the present invention has an amino acid sequence which differs from that of wild-type VWF. According to the present invention the modified VWF has at least one mutation within its D' domain, as compared to the amino acid sequence of the D' domain of wild-type VWF as shown in SEQ ID NO:31. The mutation may be a deletion, insertion or substitution. Preferably, the mutation is an amino acid substitution.
The amino acid sequence of the D’ domain of the modified VWF can have one or more mutations relative to SEQ ID NO:31. The amino acid sequence of the D’ domain of the modified VWF may have one, two, three, four, five or more mutations relative to SEQ ID NO:31. It is preferred that the amino acid sequence of the D’ domain of the modified VWF has one, two or three mutations relative to SEQ ID NO:31. Most preferably, the amino acid sequence of the D’ domain of the modified VWF has exactly one substitution relative to the amino acid sequence as shown in SEQ ID NO:31.

In a first approach, the amino acid positions which are preferably mutated in the modified VWF increase the positive charge of the D’domain and/or reduce the negative charge thereof. This first approach is referred to herein as "electrostatic approach". This can be achieved by replacing at least one amino acid having a negative charge at pH 7.4 with at least one amino acid which is neutral or has a positive charge at pH 7.4. Alternatively, this can be achieved by replacing at least one amino acid which is neutral at pH 7.4 with at least one amino acid having a positive charge at pH 7.4. These amino acid types are defined as follows:

- Amino acids having a negative charge at pH 7.4 are aspartic acid (aspartate) and glutamic acid (glutamate); they are referred to as "negatively charged amino acids" hereinafter.
- Amino acids having a positive charge at pH 7.4 are lysine and arginine; they are referred to as "positively charged amino acids" hereinafter.
- Amino acids which are neutral at pH 7.4 are alanine, glycine, asparagine, glutamine, serine, threonine, histidine, isoleucine, leucine, methionine, valine, phenylalanine, tyrosine, tryptophane, proline, and cysteine; they are referred to as "neutral amino acids" hereinafter.

In one aspect of the electrostatic approach, the invention relates to a polypeptide comprising a modified von Willebrand Factor (VWF), wherein the amino acid sequence of said modified VWF has at least one substitution within the D’ domain relative to the amino acid sequence of the D’ domain of wild type VWF as shown in SEQ ID NO:31,
wherein said substitution replaces a negatively charged amino acid present in the D' domain of wild type VWF as shown in SEQ ID NO:31 with a neutral amino acid or with a positively charged amino acid.

In another aspect of the electrostatic approach, the invention relates to a polypeptide comprising a modified von Willebrand Factor (VWF), wherein the amino acid sequence of said modified VWF has at least one substitution within the D' domain relative to the amino acid sequence of the D' domain of wild type VWF as shown in SEQ ID NO:31, wherein said substitution replaces a neutral amino acid present in the D' domain of wild type VWF as shown in SEQ ID NO:31 with a positively charged amino acid.

The modified VWF of the present invention includes, but is not limited to, the following embodiments in accordance with the electrostatic approach which can be combined with each other.

In a first embodiment in accordance with the electrostatic approach, the amino acid at position 779 of the VWF amino acid sequence is a neutral amino acid or a positively charged amino acid. The amino acid at position 779 of the VWF amino acid sequence may be lysine. Alternatively, the amino acid at position 779 of the VWF amino acid sequence may be arginine. Alternatively, the amino acid at position 779 of the VWF amino acid sequence may be a neutral amino acid.

In a second embodiment in accordance with the electrostatic approach, the amino acid at position 787 of the VWF amino acid sequence is a neutral amino acid or a positively charged amino acid. The amino acid at position 787 of the VWF amino acid sequence may be lysine. Alternatively, the amino acid at position 787 of the VWF amino acid sequence may be arginine. Alternatively, the amino acid at position 787 of the VWF amino acid sequence may be a neutral amino acid.

In a third embodiment in accordance with the electrostatic approach, the amino acid at position 793 of the VWF amino acid sequence is a positively charged amino acid. The amino acid at position 793 of the VWF amino acid sequence may be lysine.
Alternatively, the amino acid at position 793 of the VWF amino acid sequence may be arginine.

In a fourth embodiment in accordance with the electrostatic approach, the amino acid at position 794 of the VWF amino acid sequence is a positively charged amino acid. The amino acid at position 794 of the VWF amino acid sequence may be lysine. Alternatively, the amino acid at position 794 of the VWF amino acid sequence may be arginine.

In a fifth embodiment in accordance with the electrostatic approach, the amino acid at position 796 of the VWF amino acid sequence is a neutral amino acid or a positively charged amino acid. The amino acid at position 796 of the VWF amino acid sequence may be lysine. Alternatively, the amino acid at position 796 of the VWF amino acid sequence may be arginine. Alternatively, the amino acid at position 796 of the VWF amino acid sequence may be a neutral amino acid.

In a sixth embodiment in accordance with the electrostatic approach, the amino acid at position 798 of the VWF amino acid sequence is a neutral amino acid or a positively charged amino acid. The amino acid at position 798 of the VWF amino acid sequence may be lysine. Alternatively, the amino acid at position 798 of the VWF amino acid sequence may be arginine. Alternatively, the amino acid at position 798 of the VWF amino acid sequence may be a neutral amino acid.

In a seventh embodiment in accordance with the electrostatic approach, the amino acid at position 802 of the VWF amino acid sequence is a positively charged amino acid. The amino acid at position 802 of the VWF amino acid sequence may be lysine. Alternatively, the amino acid at position 802 of the VWF amino acid sequence may be arginine.

In an eighth embodiment in accordance with the electrostatic approach, the amino acid at position 818 of the VWF amino acid sequence is a neutral amino acid or a positively charged amino acid. The amino acid at position 818 of the VWF amino acid sequence may be lysine. Alternatively, the amino acid at position 818 of the VWF
amino acid sequence may be arginine. Alternatively, the amino acid at position 818 of
the VWF amino acid sequence may be a neutral amino acid.

In a ninth embodiment in accordance with the electrostatic approach, the amino acid
at position 819 of the VWF amino acid sequence is a positively charged amino acid.
The amino acid at position 819 of the VWF amino acid sequence may be lysine.
Alternatively, the amino acid at position 819 of the VWF amino acid sequence may be
arginine.

In a tenth embodiment in accordance with the electrostatic approach, the amino acid
at position 825 of the VWF amino acid sequence is a neutral amino acid or a
positively charged amino acid. The amino acid at position 825 of the VWF amino acid
sequence may be lysine. Alternatively, the amino acid at position 825 of the VWF
amino acid sequence may be arginine. Alternatively, the amino acid at position 825 of
the VWF amino acid sequence may be a neutral amino acid.

In an eleventh embodiment in accordance with the electrostatic approach, the amino
acid at position 835 of the VWF amino acid sequence is a neutral amino acid or a positively
charged amino acid. The amino acid at position 835 of the VWF amino acid
sequence may be lysine. Alternatively, the amino acid at position 835 of the VWF
amino acid sequence may be arginine. Alternatively, the amino acid at position 835 of
the VWF amino acid sequence may be a neutral amino acid.

In a twelveth embodiment in accordance with the electrostatic approach, the amino
acid at position 838 of the VWF amino acid sequence is a positively charged amino
acid. The amino acid at position 838 of the VWF amino acid sequence may be lysine.
Alternatively, the amino acid at position 838 of the VWF amino acid sequence may be
arginine.

In a thirteenth embodiment, the amino acid at position 853 of the VWF amino acid
sequence is a neutral amino acid or a positively charged amino acid. The amino acid
at position 853 of the VWF amino acid sequence may be lysine. Alternatively, the
amino acid at position 853 of the VWF amino acid sequence may be arginine.
Alternatively, the amino acid at position 853 of the VWF amino acid sequence may be a neutral amino acid.

In an alternative second approach, an amino acid in the D' domain may be replaced with a different amino acid which is evolutionarily conserved or at least polymorphic at the respective position. This second approach is referred to as "evolutionary approach" hereinafter.

Replacing with a different amino acid which is evolutionarily conserved in the sense of the invention means that a given amino acid within the D' domain which is present (i) only in humans is replaced with a different amino acid which is conserved in other species at the respective amino acid position, or (ii) which is present only in humans and some other species but not in most species is replaced with the more abundant respective amino acid which is present in most other species, or (iii) is common human polymorphism. This means that at least one residue of the VWF D' domain is replaced with a different amino acid which is present at the same position in one or more VWF polymorphs or orthologues having a D' domain different from SEQ ID NO:31.

The modified VWF of the present invention includes, but is not limited to, the following embodiments in accordance with the evolutionary approach which can be combined with eachother and with any embodiment(s) of the electrostatic approach.

In a first embodiment in accordance with the evolutionary approach, the amino acid at position 781 of the VWF amino acid sequence is a proline.

In a second embodiment in accordance with the evolutionary approach, the amino acid at position 789 of the VWF amino acid sequence is alanine, glycine, serine or valine.

In other preferred embodiments of the invention the D' domain of the modified VWF has the following sequence (SEQ ID NO:33).
SLSCRPPMVK LVCX²RA EGLX³CX⁴KTCX⁵ X⁶YX⁷LX⁸CMSX⁹G CVSGCLCPPG
MVRHX^X^RCVA LX¹⁰RCPCFHQG KX¹¹YAX¹²GETVK IGCNTCVCRX¹⁵ RKWNCTDHVC DA

The modified D' domain in the polypeptide of the present invention may have an amino acid sequence in accordance with one of the embodiments in the following table 2. Each line with an "embodiment No." represents an embodiment, wherein the D' domain in the polypeptide of the present invention has the amino acid sequence as shown in SEQ ID NO:33 with X¹ through X¹⁵ having the indicated meanings. "neut" means a neutral amino acid.
<table>
<thead>
<tr>
<th>Embodiment No.</th>
<th>x₀</th>
<th>x₁</th>
<th>x²</th>
<th>x³</th>
<th>x⁴</th>
<th>x⁵</th>
<th>x⁶</th>
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Embodiments 2.1 and 4.1-4.4 of table 2 are in accordance with the evolutionary approach, all other embodiments are in accordance with the electrostatic approach.

The amino acid positions which are preferably mutated in the modified VWF are selected from the group consisting of amino acid positions 779, 781, 787, 793, 794, 796, 798, 802, 818, 819, 825, 835, 838 and 853, wherein the numbering refers to the amino acid sequence shown in SEQ ID NO:2. That is, the D' domain of the modified VWF preferably has an amino acid substitution at one of positions 16, 18, 26, 30, 31, 39, 55, 56, 72, 75 or 90 of SEQ ID NO:31.

Preferably, the amino acid substitution in the modified VWF is at one of positions 789, 802, 818, 819 or 853 of the amino acid sequence as shown in SEQ ID NO:2. That is, the D' domain of the modified VWF preferably has one or more mutations at positions 39, 55, 56 and 90 of SEQ ID NO:31.

According to this invention the binding affinity of the polypeptide of the present invention to FVIII is higher than that of a reference polypeptide which has the same amino acid sequence except for the mutation in the D' domain.
The binding affinity of a VWF molecule to a Factor VIII molecule can be determined by a binding assay used in the art. For example, the VWF molecule may be immobilized on a solid support, increasing concentrations of Factor VIII are applied, incubated for a certain period of time, and after washing, bound Factor VIII is determined with a chromogenic assay. The affinity constant or dissociation constant may then be determined by Scatchard analysis or another suitable method. A method of determining the affinity of binding of human Factor VIII to von Willebrand Factor are described in Vlot et al. (1995), Blood, Volume 85, Number 11, 3150-3157. Preferably, however, the affinity of VWF to Factor VIII is determined as described in Example 4 of this application.

Any indication herein of affinity, including dissociation constants, preferably refers to the binding of the modified VWF of the invention, or of the polypeptide of the invention to single chain FVIII represented by the amino acid sequence as shown in SEQ ID NO:37.

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

The dissociation constant \( K_d \) of a complex of the polypeptide of the invention and the Factor VIII of SEQ ID NO:37 is typically less than 90% of the dissociation constant \( K_d \) of a complex of the reference polypeptide (e.g. the polypeptide of SEQ ID NO:32) and the Factor VIII of SEQ ID NO:37. The dissociation constant \( K_d \) of a complex of the polypeptide of the invention and the Factor VIII of SEQ ID NO:37 is preferably less than 75%, more preferably less than 50%, more preferably less than 25%, more preferably less than 10%, more preferably less than 5%, of the dissociation constant \( K_d \) of a complex of the reference polypeptide (e.g. the polypeptide of SEQ ID NO:32) and the Factor VIII of SEQ ID NO:37.

The binding affinity of the polypeptide of the present invention comprising the modified VWF to Factor VIII exceeds that of the reference polypeptide by at least 10%, preferably by at least 20%, more preferably by at least 30%, most preferably by at least 50%, more preferably by at least 75%, more preferably by at least 100%, more preferably by at least
250%, more preferably by at least 500%, more preferably by at least 1000%, more preferably by at least 10000%, most preferably by at least 100000%.

It has been found that the affinity of the polypeptide of the invention to single chain Factor VIII (e.g. represented by SEQ ID NO:37) is higher than to heterodimeric "two-chain" Factor VIII (e.g. represented by SEQ ID NO:36). Therefore, the preferred Factor VIII molecule in the complex of the invention is a single chain Factor VIII, most preferably it is the polypeptide of SEQ ID NO:37.

The reference polypeptide is a polypeptide the amino acid sequence of which is identical to that of the polypeptide of the present invention except for the mutation within the D' domain of VWF. That is, the reference polypeptide preferably has an amino acid sequence identical to that of the polypeptide of the present invention, with the proviso that the D' domain in the reference polypeptide consists of the amino acid sequence as shown in SEQ ID NO:31. In other words, the only difference in sequence between the polypeptide of the invention and the reference polypeptide lies in the amino acid sequence of the D' domain. The reference polypeptide has preferably been prepared under the same conditions as the polypeptide of the invention.

The polypeptide of the present invention may consist of the modified VWF. In another embodiment, the polypeptide of the present invention comprises a further amino acid sequence, preferably a heterologous amino acid sequence. The heterologous amino acid sequence is typically not fused to VWF in nature.

The present invention is particularly useful in cases where a VWF variant is used having an improved half-life. This can be achieved for example by fusing VWF to human serum albumin. It has been found, however, that such fusion proteins may have a reduced affinity to FVIII as compared to wild type VWF. This includes the risk that a complex of VWF fusion protein and FVIII administered to a patient may dissociate rather quickly, and the FVIII dissociated from the complex would bind to endogenous VWF. The positive effect of the complexation between a VWF with an increased half-life and FVIII, namely that also the half-life of FVIII is increased, can thus be lost if the affinity between VWF fusion protein and FVIII is too low. This problem is addressed by improving the binding of VWF to FVIII in accordance with this invention. As VWF fusion proteins are particularly at risk of having a
reduced FVIII affinity, the present invention is particularly applicable to VWF fusion proteins.

Therefore, in one embodiment, the polypeptide of the present invention comprises the modified VWF and a half-life enhancing protein (HLEP). Preferably, the HLEP is an albumin.

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.

In one embodiment the modified VWF has the following structure:

\[ N \text{- VWF - C-L1- H,} \quad \text{[formula 1]} \]

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 50, 1 to 30, 1 to 20, 1 to 15, 1 to 10, 1 to 5 or 1 to 3 (e.g. 1, 2 or 3) amino acids and which may be equal or different from each other. Usually, the linker sequences are not present at the corresponding position in the wild-type 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 VWF or the complex of the FVIII with the 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 VWF in tandem, e.g. as successive repeats.

**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.

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

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 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 VWF.

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

5 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:38 herein or albumin from other vertebrates or fragments thereof, or analogs or variants of these molecules or fragments thereof.

In particular, the proposed 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:38), 2 (amino acids 195-387 of SEQ ID NO: 38), 3 (amino acids 388-585 of SEQ ID NO: 38), 1 + 2 (1-387 of SEQ ID NO: 38), 2 + 3 (195-585 of SEQ ID NO: 38) or 1 + 3 (amino acids 1-194 of SEQ ID NO: 38 + amino acids 388-585 of SEQ ID NO: 38). 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 VWF fusion constructs of the invention may comprise at least one subdomain or domain of HA or conservative modifications thereof.
In a preferred embodiment the N-terminus of albumin is fused to the C-terminus of the amino acid sequence of the modified VWF. That is, the polypeptide of the present invention may have the structure:

\[ \text{N-mVWF-C-LI-A,} \]

wherein N is an N-terminal part of VWF, mVWF is the modified VWF as described hereinabove, C is a C-terminal part of VWF, LI is a chemical bond or a linker sequence and A is albumin as defined hereinabove.

**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-β fusion proteins were described that achieved enhanced biological activity, prolonged circulating half-life and greater solubility (WO 2006/000448). Fc-EPO proteins with a prolonged serum half-life and increased in vivo potency were disclosed (WO 2005/063808) as well as Fc fusions with G-CSF (WO 2003/076567), glucagon-like peptide-1 (WO 2005/000892), clotting factors (WO 2004/101740) and interleukin-10 (US 6,403,077), all with half-life enhancing properties.
In another embodiment, the functional half-life of polypeptide of the invention or of FVIII complexed with the polypeptide of the invention is prolonged compared to that of wild type VWF or to that of FVIII complexed with wild type VWF, or with the reference polypeptide as defined supra. The increase may be more than 15%, for example at least 20% or at least 50%. Again, such functional half-life values can be measured in vitro in blood samples taken at different time intervals from said mammal after the modified VWF or the complex of FVIII with modified VWF has been administered.

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

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

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).

Polynucleotides

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

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 VWF of the invention, or the polypeptide of the invention comprising the modified VWF. A first polynucleotide in the group may encode the N-terminal part of the modified VWF, and a second polynucleotide may encode the C-terminal part of the modified VWF.

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. Alternatively, both coding sequences are cloned into one expression vector either using two separate promoter sequences or one promoter and an internal ribosome entry site (IRES) element.
which may be used for example to direct the expression furin to enhance the generation of mature VWF.

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 VWF or a polypeptide comprising said modified VWF, which is part of this invention. The method comprises:

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

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

Expression of the 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-B1 1, 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 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 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 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)). Standard pharmaceutical formulation techniques are well known to persons skilled in the art (see, e.g., 2005 Physicians' Desk Reference®; Thomson Healthcare: Montvale, NJ, 2004; Remington: The Science and Practice of Pharmacy, 20th ed., Gennaro et al., Eds. Lippincott Williams & Wilkins: Philadelphia, PA, 2000). In particular, the pharmaceutical composition comprising the polypeptide variant of the invention may be formulated in lyophilized or stable liquid form. The polypeptide variant may be lyophilized by a variety of procedures known in the art. Lyophilized formulations are reconstituted prior to use by the addition of one or more pharmaceutically acceptable diluents such as sterile water for injection or sterile physiological saline solution.

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.

5 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.

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

Summary of the nucleotide and amino acid sequences referred to herein:

Table 3

<table>
<thead>
<tr>
<th>SEQ ID NO:</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>nucleotide sequence of DNA encoding SEQ ID NO:2</td>
</tr>
<tr>
<td>2</td>
<td>amino acid sequence of human VWF pre-propolypeptide</td>
</tr>
<tr>
<td>3-30</td>
<td>nucleotide sequences of primers, see Examples</td>
</tr>
<tr>
<td>31</td>
<td>amino acid sequence of the D' domain of human VWF</td>
</tr>
<tr>
<td>32</td>
<td>amino acid sequence of mature human VWF</td>
</tr>
<tr>
<td>33</td>
<td>amino acid sequence of the D' domain of mutated human VWF with 15 potentially modified residues</td>
</tr>
<tr>
<td>34</td>
<td>amino acid sequence of the D' domain of mutated human VWF with 11 potentially modified residues</td>
</tr>
<tr>
<td>35</td>
<td>amino acid sequence of the D' domain of mutated human VWF with 11 potentially modified residues</td>
</tr>
<tr>
<td>36</td>
<td>amino acid sequence of human Factor VIII</td>
</tr>
<tr>
<td>37</td>
<td>amino acid sequence of a mature single-chain Factor VIII</td>
</tr>
<tr>
<td>38</td>
<td>amino acid sequence of human serum albumin</td>
</tr>
</tbody>
</table>
Examples:

5 Example 1: Generation of expression vectors for VWF mutants

An expression plasmid based on pIRESpuro3 (Clontech) containing a full length VWF cDNA sequence in its multiple cloning site had been generated previously (pVWF-2448). The VWF cDNA sequence contained in this vector is displayed as SEQ ID NO:1, its corresponding protein sequence as SEQ ID NO:2.

For generating such expression vectors, the VWF cDNA may be amplified by polymerase chain reaction (PCR) using primer set VWF+ and VWF- (SEQ ID NO:3 and 4) 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 may be 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 will contain a wild-type cDNA of VWF downstream of the CMV promoter.

In order to introduce mutations in the VWF sequence site directed mutagenesis (QuickChange XL Site Directed Mutagenesis Kit, Stratagene, La Jolla, CA, USA) was applied on plasmid pVWF-2448 according to the following protocol as suggested by the kit manufacturer. Per mutagenesis reaction 5 µl of 10x reaction buffer, 1 µl of plasmid DNA pVWF-2448 (50ng), 1 µl (10pmol/µl) each of the respective two mutagenesis oligonucleotides as outlined in table 4, 1 µl dNTP Mix, 3 µl Quick-Solution, 1 µl Turbo Polymerase (2.5 U/µl) and 37 µl H2O were mixed and subjected to a polymerase chain reaction with an initial denaturation for 2 min at 95°C, 18 cycles of a) denaturation for 50 sec. at 95°C, b) annealing for 50 sec at 60°C and c) elongation for 14 min at 68°C, followed by a single terminal elongation phase of 7 min at 68°C. Subsequently 1 µl of DpnI enzyme from the kit was added and the reaction incubated for another 60 min at 37°C. After that 3 µl...
of the mutagenesis reaction were transformed into E.coli. Clones were isolated, plasmid DNA extracted and the mutations in the VWF sequences were verified by DNA sequencing.

The following table 4 lists the oligonucleotides used for mutagenesis, the respective mutations introduced and the designation of the resulting plasmids with the mutant VWF sequences.

<table>
<thead>
<tr>
<th>SEQ ID NO:</th>
<th>Oligonucleotide sequence (5’→3’)</th>
<th>VWF mutation (from x to y)</th>
<th>Designation of plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>GGTGTGTCAGCCGCTAAACCTCGGGGC</td>
<td>Asp 779 Asn</td>
<td>pRES-2462</td>
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<tr>
<td>6</td>
<td>CAGCCCGAGGTGTTAGGGGCAACA</td>
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</tr>
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<td>GTCCCGCTGACAACCTCGGGCTGAAGGG</td>
<td>Leu 781 Pro</td>
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<tr>
<td>8</td>
<td>CCCTTCAGCCCGAGGGTGTACCGCGGAC</td>
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</tr>
<tr>
<td>9</td>
<td>CTGAAGGGCTCGAGTGTGCAAACGGTGCCAGAAC</td>
<td>Thr 789 Ala</td>
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<tr>
<td>10</td>
<td>GTTCTGACCAGTTTGGCACACTCGAGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>GTGTACAAACGGTGCCGGAACATATGACCTTGAGTGC</td>
<td>Gin 793 Arg</td>
<td>pRES-2465</td>
</tr>
<tr>
<td>12</td>
<td>GCCACTCCAGTGCAATTCCCGGCAGCTTGTACAC</td>
<td>Asn 794 Lys</td>
<td>pRES-2466</td>
</tr>
<tr>
<td>13</td>
<td>GTACCAAAAAACGTCGCGAAGTATGACCTGGAGTGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>GCACCTCCAGTGCTACTTTTGGCACAGTTTGGTAC</td>
<td>Met 802 Arg</td>
<td>pRES-2467</td>
</tr>
<tr>
<td>15</td>
<td>CTGGAGTGCACTGAGCGAGGGTCTGTC</td>
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<td></td>
</tr>
<tr>
<td>16</td>
<td>CAGCCACAGACACAGCCCGCTGCAGCTAGCTCGAGCAGCACTCCAG</td>
<td>Met 802 Lys</td>
<td>pRES-2468</td>
</tr>
<tr>
<td>17</td>
<td>CTGGAGTGCACTGAGCGAGGGTCTGTC</td>
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<td></td>
</tr>
<tr>
<td>SEQ ID NO:</td>
<td>Oligo-nucleotide</td>
<td>Mutagenesis oligonucleotide sequence (5'→3')</td>
<td>VWF mutation (from x to y)</td>
</tr>
<tr>
<td>-----------</td>
<td>------------------</td>
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<tr>
<td>18</td>
<td>We4083</td>
<td>CAGCCAGAGACACAGCCCTTGCTCATGCACTCCAG</td>
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<tr>
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<tr>
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<tr>
<td>21</td>
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<td>We4089</td>
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<td>GCTTCCATCCGGCAAGCAGTATGCCCCGGCATGAGAAGAC</td>
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</table>

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 mutation of other amino acid residues.

**Example 2 : Transfection of plasmids and expression of VWF mutants in HEK-293 cells**
Expression plasmids were grown up in E.coli TOP10 (Invitrogen, Carlsbad, CA, USA) and purified using standard protocols (Qiagen, Hilden, Germany). HEK-293 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. Transfected cell populations were spread through T-flasks into shake flasks from which supernatants were harvested for VWF antigen quantitation and Biacore analysis.

**Example 3: Quantitation of VWF antigen**

VWF antigen in culture supernatant 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 rmll/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 4: Analysis of the binding of VWF mutants to FVIII

All binding tests are performed using a Biacore 3000 instrument (GE Healthcare) and CM 3 chips. System buffer and dilution buffer for FVIII products is HBS-P (20 mmol/L Hapes, 100 mmol/L NaCl, 0.005% polysorbate 20, pH 7.3). A monoclonal anti-vWF antibody not interfering with FVIII binding is immobilized by using Biacore amino coupling chemistry. All immobilization, saturation and binding assays are performed at a controlled temperature of 25°C.

Monoclonal anti-vWF antibody is covalently bound to an activitated CM 3 chip by NHS and EDC (both from GE Healthcare), a coupling where the antibody is fixed at its aminoterminus to the dextran filaments on the gold surface of the chip. For immobilization the monoclonal antibody is diluted to 10 µg/mL in 10 mM sodium acetate (pH 4.5). The antibody solution is flown over the chip for 8 min at a flowrate of 5 µL/min.

After the immobilization procedure non-coupled dextran filaments are saturated by flowing 1M ethanolamine (pH 8.3) over the chip for 5 min (at a flow rate of 5 µL/min). A reference flow cell is set up by saturating an empty flow cell with ethanolamine by using the same procedure as above.

VWF mutants are immobilized to the covalently coupled anti VWF monoclonal antibody by flowing VWF mutants (in culture supernatant) over the chip until its saturation at a flow rate of 5 µL/min.

For evaluation of the binding of VWF mutants to FVIII, a FVIII preparation is serially diluted in HBS-P buffer, e.g. to concentrations 0.3125 µg/mL, 0.625 µg/mL, 1.25 µg/mL, 2.5 µg/mL and 5 µg/mL. A sample of each dilution is flown over the chip for 12 min (flow rate 10 µL/min), followed by a dissociation time of 5 min with HBS-P buffer. After each run the chip is washed with 250 mM CaCl₂ for 3 min. to elute FVIII bound to VWF. Thereafter the VWF mutant is stripped by washing with 10mM glycine (pH 2.1) for 4 min and a the next VWF mutant is bound to the chip as described above.

Binding parameters are calculated using BIAevaluation Software (Biacore, GE Healthcare). The curve fitting methods are based on Langmuir equations. The input data for calculations
are the molar masses of the analytes, other parameters like max. RU and slopes are automatically extracted out of the fitted association and dissociation curves. The outputs of Biaevaluation Software are the association rate constants and the dissociation rate constants, from which the affinity constants are calculated.
5 Claims

1. A polypeptide comprising a modified von Willebrand Factor (VWF), wherein the amino acid sequence of said modified VWF has at least one mutation within the D' domain relative to the amino acid sequence of the D' domain of wild type VWF as shown in SEQ ID NO:31, and wherein the binding affinity of said polypeptide comprising a modified VWF to Factor VIII (FVIII) is higher than that of a reference polypeptide, wherein the amino acid sequence of said reference polypeptide is identical to the amino acid sequence of said polypeptide comprising a modified VWF except that the amino acid sequence of the D' domain of the reference polypeptide is identical with SEQ ID NO:31.

2. The polypeptide of claim 1, wherein the Factor VIII binding affinity of the polypeptide exceeds that of the reference polypeptide by at least 10 percent.

3. The polypeptide of claim 1 or 2, wherein the dissociation constant $K_D$ of a complex of the polypeptide and the Factor VIII of SEQ ID NO:37 is less than 90% of the dissociation constant $K_D$ of a complex of the reference polypeptide and the Factor VIII of SEQ ID NO:37.

4. The polypeptide of any one of claims 1 to 3, wherein said at least one mutation within the D' domain includes an amino acid substitution where the FVIII binding affinity has been increased by (i) replacing at least one acidic residue of the VWF D' domain with a neutral or basic amino acid, (ii) by replacing at least one neutral residue of the VWF D' domain with a basic amino acid, and/or (iii) by replacing at least one residue of the VWF D' domain with a different amino acid which is present at the same position in one or more VWF polymorphs or orthologues having a D' domain different from SEQ ID NO:31.

5. The polypeptide of any one of claims 1 to 4, wherein said at least one mutation within the D' domain includes an amino acid substitution at one of positions 779, 781, 787,
789, 793, 794, 796, 798, 802, 818, 819, 825, 835, 838 and 853 of the VWF amino acid sequence as shown in SEQ ID NO:2.

6. The polypeptide of any one of claims 1 to 4, wherein the polypeptide comprises an amino acid sequence as shown in SEQ ID NO:33 or SEQ ID NO:34.

7. The polypeptide of claim 5, wherein said amino acid substitution within the D' domain is selected from the group consisting of Asp779Asn, Leu781 Pro, Thr789Ala, Gln793Arg, Asn794Lys, Met802Arg, Met802Lys, Glu818Ala, Glu818Lys, Asn819Lys, Glu835Gln, Pro838Lys, and Asp853Asn, wherein the numbering refers to SEQ ID NO:2.

8. The polypeptide of any one of claims 1 to 7, further comprising a half-life enhancing protein (HLEP).

9. The polypeptide of claim 8, wherein said HLEP is an albumin.

10. The polypeptide of claim 9, wherein the N-terminus of the albumin is fused to the C-terminus of the VWF amino acid sequence.

11. A complex comprising a Factor VIII molecule and the polypeptide of any one of claims 1 to 10.

12. The complex of claim 11 wherein the Factor VIII is the polypeptide of SEQ ID NO:37, and wherein the dissociation constant $K_D$ of the complex is less than 90% of the dissociation constant $K_D$ of a complex of the reference polypeptide and the Factor VIII of SEQ ID NO:37.

13. The polypeptide of any one of claims 1 to 12 or the complex of claim 11 or 12 for use in the treatment or prophylaxis of a bleeding disorder.

14. The polypeptide or complex for use according to claim 13, wherein the bleeding disorder is von Willebrand's disease (VWD) or hemophilia A.
15. A pharmaceutical composition comprising the polypeptide of any one of claims 1 to 10 or the complex of claim 11 or 12.

16. A method of treating a bleeding disorder, comprising administering to a patient in need thereof, a pharmaceutically effective amount of the polypeptide of any one of claims 1 to 10 or of the complex of claim 11 or 12.

17. The method of claim 16, wherein the bleeding disorder is von Willebrand's disease (VWD) or hemophilia A.

18. A polynucleotide encoding the polypeptide of any one of claims 1 to 10.

19. A plasmid or vector comprising the polynucleotide of claim 18.

20. The plasmid or vector of claim 19, said plasmid or vector being an expression vector.

21. A host cell comprising the polynucleotide of claim 18 or the plasmid of claim 19 or 20.

22. A method of producing a polypeptide comprising a modified VWF, comprising: (a) culturing the host cells of claim 21 under conditions such that the polypeptide comprising a modified VWF is expressed; and (b) optionally recovering the polypeptide comprising a modified VWF from the host cells or from the culture medium.

23. A method of increasing the Factor VIII binding affinity of VWF, comprising introducing a mutation into the D' domain of the VWF amino acid sequence, said mutation not being present in the amino acid sequence of the D' domain of wild type VWF as shown in SEQ ID NO:31.

24. The method of claim 23, wherein at least one acidic residue of the VWF D' domain is replaced with a neutral or basic amino acid, or wherein at least one neutral residue of the VWF D' domain is replaced with a basic amino acid.
25. The method of claim 23, wherein at least one residue of the VWF D' domain is replaced with a different amino acid which is present at the same position in one or more VWF polymorphs or orthologues having a D' domain different from SEQ ID NO:31.

26. The use of a modified VWF having a higher affinity to FVIII than non-modified VWF for increasing the half-life of FVIII.

27. The use of claim 26, wherein said modified VWF is the polypeptide of any one of claims 1 to 10, or the modified VWF as defined in any one of claims 1 to 8.

28. The use of claim 26 or 27, wherein said modified VWF is a fusion protein, preferably an albumin fusion protein.

29. A method of preparing a complex comprising FVIII and VWF, comprising mixing a FVIII with the polypeptide of any one of claims 1 to 10.
**INTERNATIONAL SEARCH REPORT**

**A. CLASSIFICATION OF SUBJECT MATTER**

INV. A61K38/36 C07K14/755 C12N15/63

ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

A61K C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, Sequence Search, EMBASE, WPI Data

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

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<td>10 January 2008 (2008-01-10) claims 1-74</td>
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Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents :
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  * E earlier application or patent but published on or after the international filing date
  * L document which may throw doubts on priority claim(s) on which the later document is considered to be of particular relevance
  * O document referring to an oral disclosure, use, exhibition or other special reason (as specified)
  * P document published prior to the international filing date but later than the priority date claimed

**Date of the actual completion of the international search**

11 March 2013

**Date of mailing of the international search report**

22/03/2013

**Name and mailing address of the ISA/Authorized officer**

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<td>VOORBERG J ET AL: &quot;DOMAINS INVOLVED IN MULTIMER ASSEMBLY OF VON WILLEBRAND FACTOR VWF MULTIMERIZATION IS INDEPENDENT OF DIMERIZATION&quot;, EMBO (EUROPEAN MOLECULAR BIOLOGY ORGANIZATION) JOURNAL, vol. 9, no. 3, 1990, pages 797-803, XP002674469</td>
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