von Willebrand factor (vWF)-containing Preparations, and Methods, Kits, and Uses Related Thereto

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims the benefit of U.S. Application No. 61/261,145 filed Nov. 13, 2009, which is herein incorporated by reference in its entirety.

FIELD OF THE INVENTION

Methods, compositions and kits are provided for vWF-containing preparations, including methods, kits and use of such preparations for preparing Factor VIII (FVIII). Also provided are vWF polypeptides and nucleic acid molecules encoding same.

BACKGROUND OF THE INVENTION

FVIII expressed by mammalian cells is often specifically or non-specifically absorbed onto cell surfaces by interaction with surface components (*e.g.* proteoglycans) or by receptor-mediated events (*e.g.* interaction with LRP receptor). It is also possible that expressed FVIII is enzymatically cleaved and/or degraded in the media of cultured cells. Over time in culture, expressed FVIII concentration decreases in media unless the secreted material is rapidly removed after expression (*e.g.* by perfusion techniques).

Under ordinary circumstances, the FVIII-vWF complex may be removed from media by conventional chromatographic methods including absorption onto charge matrices or by pseudo-affinity chromatography. FVIII can then be purified away from the FVIII:vWF complex by selective washing steps to yield an enriched population of FVIII molecules, minimally contaminated by vWF.

vWF is formed in the vascular endothelial cells, which are the main source of this plasma protein, by constitutive or stimulated liberation, but it is also synthesized in smaller amounts by the megakaryocytes. It is believed that the primary product of translation is comprised of 2813 amino acids. After cleaving off the signal peptide (22 amino acids), dimerization takes place. Further processing is effected in the Golgi apparatus, the dimers polymerizing after cleavage and removal of the propeptide (741 amino acids). The propeptide plays an important role in the further linking of the dimers, where it catalyses the formation of disulfide bridges at the amino-

terminal end. Thus, differently sized oligomers ranging in size from a dimer of 500,000 daltons to large multimers of up to 20 million daltons may form. In addition to the proteolytic procedures, vWF is subject to other post-translational modifications, including glycosylation and sulfation.

SUMMARY OF THE INVENTION

In one aspect, the present invention provides a polypeptide comprising a first amino acid sequence present in a von willebrand factor
(vWF) polypeptide and a second amino acid sequence heterologous to the first, wherein the polypeptide is capable of binding a Factor VIII (FVIII), with the proviso that the polypeptide lacks von willebrand factor (vWF) domains D4, B1, B2, B3, C1, C2 and CK, wherein the second amino acid sequence is C-terminus to the first amino acid sequence, , wherein the first amino acid sequence is as set forth in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14 or SEQ ID NO:15, and wherein the second amino acid sequence corresponds to an immunoglobulin Fc.

In one aspect, the present invention provides a composition comprising the polypeptide as defined above.

In one aspect, the present invention provides a protein complex comprising the polypeptide as defined above, and a Factor VIII (FVIII) polypeptide.

In one aspect, the present invention provides a composition comprising the protein complex as defined above.

In one aspect, the present invention provides a nucleotide sequence encoding the polypeptide as defined above.

In one aspect, the present invention provides an expression vector comprising the nucleotide sequence as defined above.

In one aspect, the present invention provides a cell expressing the polypeptide as defined above.

In one aspect, the present invention provides a cell expressing the protein complex as defined above.

In one aspect, the present invention provides an in vitro method for preparing the protein complex as defined above, the method comprising contacting the polypeptide with the Factor VIII (FVIII).

In one aspect, the present invention provides an in vitro method for binding a Factor VIII (FVIII), the method comprising: contacting a Factor VIII (FVIII) polypeptide with a polypeptide as defined above to form a protein complex comprising the polypeptide as defined above and the Factor VIII (FVIII).

In one aspect, the present invention provides the use of a composition comprising the protein complex as defined above, in the manufacture of a medicament for enhancing a plasma pharmacokinetic property of a Factor VIII (FVIII).

In one aspect, the present invention provides a composition comprising the protein complex as defined above, and a pharmaceutically acceptable carrier.

In one aspect, the present invention provides the use of the composition as defined above in the manufacture of a medicament for treating hemophilia.

In one aspect, the present invention provides a polypeptide comprising a first amino acid sequence present in a vWF polypeptide and a second amino acid sequence heterologous to the first, wherein the polypeptide is capable of binding a FVIII.

In another aspect, the present invention provides a composition comprising the polypeptide comprising the first and the second amino acid sequence.

In some aspects, the present invention provides a protein complex comprising the polypeptide and the FVIII.

In other aspects, the present invention provides a composition comprising the protein complex.

In still further aspects, the present invention provides a nucleotide sequence encoding the polypeptide comprising the first and the second amino acid sequence.

In one aspect, the present invention provides an expression vector comprising the nucleotide sequence.

In another aspect, the present invention provides a cell expressing the polypeptide comprising the first and the second amino acid sequence.

In some aspects, the present invention provides a cell expressing the protein complex comprising the polypeptide and the FVIII.

In other aspects, the present invention provides a method for preparing the protein complex, the method comprising contacting the polypeptide with the FVIII.

In one aspect, the present invention provides a method for preparing a FVIII, the method comprising: contacting the FVIII with the polypeptide comprising the first and the second amino acid sequence to form a protein complex comprising the polypeptide and the FVIII.

In another aspect, the present invention provides a method for enhancing a plasma pharmacokinetic property of a FVIII, the method comprising administering to a subject a composition comprising the protein complex comprising the polypeptide and the FVIII.

In some aspects, the present invention provides a composition comprising the protein complex, and a pharmaceutically acceptable carrier.

In other aspects, the present invention provides a method for treating a blood condition, the method comprising administering the composition comprising the protein complex comprising the polypeptide and the FVIII, wherein the polypeptide comprises the first and the second amino acid sequence.

In still further aspects, a kit is provided.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows a schematic representation of human von Willebrand Factor protein structure, processing and maturation. (A) Domain structure of the primary von Willebrand Factor polypeptide: SS = signal peptide; D1 and D2 = propeptide sequence; D'-D3 = includes nominal Factor VIII binding region; A1 and A3 = collagen binding domains (and other interactions); (B) During secretion and processing, the signal peptide is removed and the propeptide is later cleaved from the vWF polypeptide by a furin-like processing step to yield a mature vWF polypeptide that initiates normally at the junction of the D' domain; (C) Propeptide associated with the mature vWF polypeptide promotes increased FVIII binding and multimerization; and (D) Cysteine residues provided by the propeptide-matured vWF complex provide covalent bridges that allow for intra- and inter-molecular multimer formation.

Figure 2 shows a schematic representation of human von Willebrand Factor domain truncations with covalent fusion to IgG_1 Fc. (A) Domain structure of the primary von Willebrand Factor polypeptide: SS = signal peptide; D1 and D2 = propeptide sequence; D'-D3 = includes the nominal Factor VIII binding region; A1 and A3 = collagen binding domains (and other interactions); (B) The primary vWF truncation polypeptides with Fc are diagrammed, showing the expected domain structure after signal peptide cleavage. Sequences include the propeptide followed by either the D'-D3, the D'-A1 or the D'-A3 domain, each in turn fused covalently at the hinge region of the constant region of IgG_1; and (C) Propeptide associated with vWF truncation polypeptide-Fc fusions (*i.e.*, D'-D3-Fc, D'-A1-Fc, or D'A3-Fc) promotes increased FVIII binding and multimerization, just as occurs in mature vWF processing.

Figure 3 shows a schematic representation of truncated vWF-Fc fusion processing, maturation and multimerization of the primary translation product. (A) Representation of

propeptide and truncated vWF domain-Fc fusion after signal peptide cleavage. In this representation, the polypeptides are derived from a single primary translation product as depicted in Figure 1, for example; (B) Representation showing propeptide and vWF domain-Fc fusion monomer after processing of primary translation product and association of propeptide with truncated vWF domain-Fc polypeptide to promote proper folding; and (C) Cysteine residues contained within the hinge region of the IgG_1 Fc provide the intra-molecular bridging to help create a functional vWF-Fc dimer that also binds FVIII, in a manner similar to that found in plasma-derived vWF; vWF propeptides, in turn, promote multimerization of the fusion polypeptides.

Figure 4 shows a schematic representation of truncated vWF-Fc fusion processing, maturation and multimerization of two primary translation products from independent coding regions. (A) Representation of separate and independent primary translation products of propeptide and of truncated vWF domain-Fc fusion polypeptides after signal peptide cleavage; no furin-like processing is needed as in normal vWF processing. In this representation, the polypeptides are derived from two independent primary translation products from two independent promoter cassettes either transcribed from one expression vector or from two expression vectors co-expressed in the same cell, the former expression mode depicted in Figure 6, for example; (B) Representation showing propeptide and vWF domain-Fc fusion monomer after processing of primary translation product and association of co-expressed propeptide with truncated vWF domain-Fc polypeptide to promote proper folding; and (C) Cysteine residues contained within the hinge region of the IgG_1 Fc provide the intra-molecular bridging to help create a functional vWF-Fc dimer that also binds FVIII, in a manner similar to that found in plasma-derived vWF; vWF propeptides, in turn, promote multimerization of the fusion polypeptides.

Figure 5 is a schematic illustrating, in some embodiments, transfection of plasmid expression vectors into mammalian cells that express mature vWF or truncated vWF domain-Fc fusion polypeptides. (A) Expression plasmid with coding sequence for vWF or truncated vWF domain-Fc fusion proteins, each containing signal peptide sequences and propeptide domains as part of the primary translation product; (B) Representation of plasmids transfected and taken up into mammalian cells under selection to create a stable expressing cell line; (C) Expression plasmid with coding cassette for Factor VIII using a different selectable marker (neomycin) than

in (A); and (D) Representation of FVIII (C) and vWF or vWF-Fc (D) plasmids co-transfected and taken up into mammalian cells under selection to create a stable cell line that expresses FVIII and vWF-Fc (or vWF).

Figure 6 is a schematic illustrating, in other embodiments, transfection of plasmid expression vectors into mammalian cells that express mature vWF or truncated vWF domain-Fc fusion polypeptides and propeptide sequences from independent promoters, either on the same or different plasmid vectors. (A) Expression plasmid with coding cassettes for vWF or truncated vWF domain-Fc fusion proteins that do not express a propeptide domain as part of their primary translation product, and for the vWF propeptide domain expression cassette (each with respective signal peptide sequences) from independent promoters; (B) Representation of propeptide-minus vWF or vWF-Fc fusion polypeptides and propeptide polypeptide plasmids transfected and taken up into mammalian cells under selection to create a stable cell line that co-expresses both the vWF or vWF-Fc proteins as well as the propeptide polypeptide; (C) Expression plasmid with coding cassette for Factor VIII using a different selectable marker (neomycin) than in (A); and (D) Representation of FVIII (C) and vWF or vWF-Fc with propeptide (D) plasmids co-transfected and taken up into mammalian cells under selection to create a stable cell line that expresses FVIII, and either vWF-Fc or vWF, and independently, the vWF propeptide.

Figure 7 shows Coomassie stained proteins of expressed proteins electrophoresed on 4-12% Bis-Tris PAGE gels under reducing and denaturing conditions, either directly from expressed culture supernatants (A) or from Protein G immunopreciptations (B) of the proteins present in the cultured supernatants from (A). PER.C6 cells transfected with plasmids containing a truncated vWF-Fc construct were selected for stable cultures as pool using antibiotic selection. For direct supernatant samples, 20 microliters were loaded onto a gel. For immunoprecipitation, 20 microliters of Protein G beads were added to either 1 ml of medium with D'-D3-Fc, or to 0.2 ml of medium with D'-A1-Fc or D'-A3-Fc. Lanes on both gels represent: Lane 1: Molecular weight standard; Lane 2: D'-D3-Fc; Lane 3: pro-D'-D3-Fc; Lane 4: D'-A1-Fc; Lane 5: pro-D'-A1-Fc; Lane 6: D'-A3-Fc; Lane 7: pro-D'-A3-Fc; and Lane 8: untransfected PER.C6 conditioned medium as control. Higher molecular weight bands in lanes 3, 5 and 8 represent unprocessed propeptide still attached to the corresponding vWF domains.

Figure 8 is a bar chart showing recovered FVIII activity using vWF-Fc fusion proteins or plasma-derived vWF protein. The dashed bar indicates the increased recovery above the control (*i.e.*, BDD-FVIII without added vWF proteins). Cell supernatants containing expressed, truncated vWF-Fc fusion proteins or full-length vWF were mixed with BDD-078 cells expressing recombinant FVIII. After two days, samples were analyzed for FVIII expression using a chromogenic FVIII assay.

Figure 9 is a gel showing multimerization of pro-vWF-Fc fusion proteins, with a comparison to normal, plasma-derived multimers of vWF. Plasma-derived factor VIII (Koate-DVI®) was run as a standard for multimerization on denaturing, but not reducing, 1.6% (lane 1) and 2% HGT(P) (lane2) agarose gels, while samples of pro-D'-D3-Fc (lane 3), pro-D'-A1-Fc (lane 4) and pro-D'-A3-Fc (lane 5) proteins were electrophoresed on 1.6% gels to visualize the differences in ladder sizes. Brackets identify the position of vWF dimer triplets in lane 1 and corresponding position in lane 2. As expected, the increasing sizer of the pro-vWF-Fc polypeptide chain results in creation of multimer bands of increasing molecular weight, in the order: pro-D'-D3-Fc < pro-D'-A1-Fc < pro-D'-A3-Fc.

Figure 10 shows purification of FVIII from supernatants containing pro-D'A3-Fc/FVIII complexes: (A) Chromatographic trace of peaks eluted during different buffer conditions; and (B) Coomassie-stained 7.5% PAGE gel with samples taken from the column trace represented in (A) shows the specific elution of FVIII from the pro-D'A3-Fc/FVIII complexes in lane 4. Lane Lanes 1-9 represent, respectively, (1) starting material, (2) flow-through , (3) 0.1M $CaCl_2$ eluate, (4) 0.3M $CaCl_2$ eluate, (5) pH 5.5 citrate eluate, (6) a concentrated BDD-FVIII preparation, (7) molecular weight markers (sizes on right side), (8) cell supernatant from pro-D'-A3-Fc expressing PER.C6 cells, (9) commercial BDD-FVIII (Xyntha®), asterisks showing three bands of approximately 170, 90, and 80 kd, corresponding to full-length BDD-FVIII, heavy chain and light chain(s), respectively.

Figure 11 shows purification of FVIII from supernatants containing commercial recombinant B-domain-deleted FVIII (Xyntha) with supernatant containing pro-D'D3-Fc protein After elution from gels with different buffer compositions, fractions were analyzed on 7% NuPAGE reducing/denaturing polyacrylamide gels and stained with Coomassie Brilliant Blue. Lane 7 shows essentially pure FVIII recovered after elution from pro-D'-D3-Fc/FVIII complexes bound to Protein A. Lanes 1-9 represent, respectively, (1) molecular weight polypeptide markers

with molecular weights listed to the left, (2) pro-D'-D3-Fc cell supernatant, (3) commercial B-domain-deleted FVIII (Xyntha®), (4) Xyntha® mixed with pro-D'-D3 supernatant, (5) supernatant (pro-D'D3-Fc + Xyntha®) load onto Protein A column, (6) flow-through wash with 20 mM Tris-HCl, pH 7.0, (7) 0.3M $CaCl_2$ eluate, and (8) pH 3.9 glycine wash that acts to strip the column of additional Protein A-binding proteins. The three asterisks in lane 3 align with proteins bands of approximately 170, 90, and 80 kd, corresponding to full-length BDD-FVIII, heavy chain and light chain(s), respectively.

DETAILED DESCRIPTION

In one aspect, the present invention provides a polypeptide comprising a first amino acid sequence present in a vWF polypeptide and a second amino acid sequence heterologous to the first, wherein the polypeptide is capable of binding a FVIII.

As used herein, the term "capable of binding" contemplates embodiments wherein the capability of the polypeptide to bind to the FVIII is effected by higher order protein assembly and/or one or more post-translational modifications such as, for example, signal peptide cleavage, propeptide cleavage, propeptide association, phosphorylation, glycosylation, and such like. For example, in some embodiments, the polypeptide is "capable of binding" to the FVIII as a dimer, trimer, tetramer, or higher order multimeric complex that forms subsequent to multimerization of the polypeptide. Or, for example, in other embodiments, the polypeptide is "capable of binding" the FVIII following multimerization of the polypeptide subsequent to association of a propeptide with the polypeptide. "Multimerization" and "oligomerization" are used interchangeably herein and refer to the association two or more protein molecules, mediated by covalent (*e.g.,* intermolecular disulfide bonds) and/or non-covalent interactions. Accordingly, "multimer(s)" and "oligomer(s)" also are used interchangeably herein.

vWF and FVIII polypeptides of human and non-human (*e.g.,* primates, dogs, cats, horses, pigs, mice, rats, guinea pigs, rabbits, cows, other vertebrates) origin are contemplated by the present invention including natural, synthetic, and recombinant proteins. Also within the scope of the present invention are vWF and FVIII polypeptides corresponding to wild-type proteins, or mutants, variants, and/or truncations thereof. For example, in some embodiments, the first amino acid sequence corresponds to a fragment of a vWF polypeptide of human origin, wherein

the heterologous second amino acid sequence comprises or consists of a sequence not present in any vWF protein, human or otherwise. FVIII and/or vWF include native proteins, as well as derivative thereof, *e.g.* proteins mutated by deletion, substitution or insertion, or a chemical derivative or fragment thereof.

I. First Amino Acid Sequence

In one embodiment, the first amino acid sequence defines a structure or domain that reacts with a monoclonal anti-vWF antibody capable of specifically binding to a region of a VWF polypeptide comprising a FVIII binding domain. In one embodiment, the monoclonal antibody is monoclonal antibody C3 as described by, *e.g.,* Foster *et al.*, JBC, 262:8443 (1987) and Fulcher *et al.*, J. Clin. Invest., 76:117 (1985), each of which is herein incorporated by reference for its teaching of monoclonal antibody C3 and method of preparing monoclonal antibodies, in particular monoclonal antibody C3.

Non-limiting examples of vWF amino acid sequences and nucleic acid sequences encoding vWF or a portion thereof are disclosed by, *e.g.,* GenBank Accession Nos.: NP_000543, NM_000552, AAE20723, AAB59512, P04275, EAW88815, ACP57027, EAW88816, and AAD04919; U.S Patent No. 5,260,274; Titani *et al.*, Biochemistry, 25:3171-84 (1986); and Sadler *et al.*, PNAS, 82:6391-6398 (1985), each of which is herein incorporated by reference for its teaching of amino acid and nucleic acid sequences corresponding to vWF.

A person of ordinary skill in the art knows that the prototypical preprop-vWF is a polypeptide of 2813 amino acids with a signal peptide of 22 amino acids and repetitive functional domains, A, B, C, D and CK, which are distributed from the amino terminal in the order "D1," "D2," "D'," "D3," "A1," "A2," "A3," "D4," "B1," "B2," "B3 (the latter three collectively considered "B"), "C1," "C2," and "CK." The "mature" vWF subunit is composed of, from the N- to the C-terminus in the order, the domains: D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-CK.

An amino acid sequence of an exemplary full-length human vWF is shown by SEQ ID NO:29, which is encoded by nucleotides 251-8689 of SEQ ID NO:30. With reference to SEQ ID NO:29, the "signal peptide" portion of vWF spans amino acid positions 1 though Cys-22, the "propeptide" portion (D1-D2) spans amino acid positions 23 through Arg-763, and the "mature" vWF protein spans amino acid positions 764 through 2813. The individual domains have also been approximately mapped as D': 764 – 865; D3: 866 – 1242; A1: 1260 – 1479; A2: 1480 –

1672; A3: 1673 – 1874; D4: 1947 – 2298; B: 2296 – 2399; C1: 2400 – 2516; C2: 2544 – 2663; and CK: 2720 – 2813. An alternative vWF domain mapping and naming system has been used by the EXPASY Protein Database convention (worldwideweb.uniprot.org/uniprot/P04275) as D1: 34 – 240; D2: 387 – 598; D': 776 – 827; D3: 866 – 1074; A1: 1277 – 1453; A2: 1498 – 1665; A3: 1691 – 1871; D4: 1949 – 2153; B: 2255 – 2328 (which is named C1 in EXPASY); C1: 2429 – 2495 (named C2 in EXPASY); C2: 2580 – 2645 (named C3 in EXPASY); and CK: 2724 – 2812.

Non-limiting examples of FVIII amino acid and nucleic acid sequences are disclosed by, *e.g.*, GenBank Accession nos. 1012296A AAA52420.1, CAA25619.1, AAA52484.1, 1012298A, EAW72647.1, EAW72646.1, XP_001498954.1, ACK44290.1, ACO95359.1, NP_001138980.1, ABZ10503.1, NP_032003.2, U.S. Patent No. 6,307,032, and Wood *et al.*, Nature, 312:330-7 (1984), each of which is herein incorporated by reference for its teaching of FVIII sequences. Variants, derivatives, modifications, and complexes of FVIII also are known in the art, and are encompassed in the present invention. For example, variants of Factor VIII, as described in, U.S. Pat. No. 5,668,108 discloses variants of FVIII whereby the aspartic acid at position 1241 is replaced by a glutamic acid with the accompanying nucleic acid changes as well; U.S. Pat. No. 5,149,637 describes FVIII variants comprising the C-terminal fraction, either glycosylated or unglycosylated; and U.S. Pat. No. 5,661,008 describes a FVIII variant comprising amino acids 1-740 linked to amino acids 1649 to 2332 by at least 3 amino acid residues; each of which is herein incorporated by reference for each teaching of FVIII variant sequences.

In one embodiment, the FVIII is plasma- or serum-derived FVIII. In another embodiment, the FVIII is recombinant FVIII, *e.g.* active human FVIII expressed in cultured mammalian cells from recombinant DNA clones. Expression systems for the production of Factor VIII are known in the art, and include prokaryotic and eukaryotic cells, as exemplified by U.S. Pat. Nos. 5,633,150, 5,804,420, and 5,422,250, each of which is incorporated herein for its teaching of production of FVIII.

One of ordinary skill in the art knows that the ability of the polypeptide to bind FVIII may be determined in a variety of ways. In particular, the polypeptide of the present invention may be assayed for its ability to bind the FVIII using techniques described herein and/or adapting techniques known in the art. For example, to analyze/determine binding, immunoassays can be employed including, but are not limited to, competitive and non-

competitive assay systems using techniques such as western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, etc. (see, *e.g.,* Ausubel *et al.*, eds, 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York, which is herein incorporated by reference in its entirety).

For example, the polypeptide comprising the first and the second amino acid sequences can be contacted with the FVIII in a suitable buffer such as TBS in the presence of a monoclonal antibody coupled to Sepharose. The antibody can be directed against a region of the polypeptide such that binding of the antibody to the polypeptide does not interfere with binding of the polypeptide with the FVIII (*e.g.,* the antibody may be directed against the second amino acid sequence or an "A1", or "A2" or "A3" repeat region of vWF where such region also is present on the polypeptide Following contact, FVIII bound to the polypeptide/antibody and unbound FVIII can be separated, *e.g.* by centrifugation, and FVIII can be measured, *e.g.* using a chromogenic substrate assay (Factor VIII Coatest; Chromogenix, Mölndal, Sweden).

In preferred embodiments, the first amino acid sequence of the polypeptides of the present invention is a truncated vWF polypeptide. For example, truncated forms of vWF, in some embodiments, include (i) truncated vWF polypeptides that lack the "propeptide" sequence; and (ii) truncated vWF polypeptides that lack the "A1," "A2," "A3," "D4," "B" (also known as "B1", "B2", and "B3"), "C1," "C2," and/or "CK" domain of the mature sequence. Other truncated or otherwise modified forms of vWF also are contemplated.

In one embodiment, the first amino acid sequence is as set forth in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:33, SEQ ID NO:34, or SEQ ID NO:35.

II. Second Amino Acid Sequence

In other embodiments, the second amino acid sequence of the polypeptide provides a structure or domain having affinity for a binding partner.

The second amino acid sequence is heterologous to the first. In one embodiment, the heterologous second amino acid sequence comprises or consists of a sequence not present in any vWF protein. In one embodiment, at least a portion (*e.g.,* a contiguous portion) of the

heterologous second amino acid sequence corresponds to a sequence not present in any vWF polypeptide.

Preferably, in some embodiment, the second amino acid sequence corresponds to an antibody Fc polypeptide such as, *e.g.,* a human IgG1 Fc region. For example, the second amino acid sequence can correspond to the amino acid residues that extend from the N-terminal hinge region to the native C-terminus, *i.e.,* is an essentially full-length antibody Fc region. Fragments of Fc regions, *e.g.,* those that are truncated at the C-terminal end, also may be employed. In some embodiments, the fragments preferably contain one or more cysteine residues (at least the cysteine residues in the hinge region) to permit interchain disulfide bonds to form between the Fc polypeptide portions of two separate polypeptides of the present invention, forming dimers.

Other antibody Fc regions may be substituted for the human IgG1 Fc region. For example, other suitable Fc regions are those that can bind with affinity to protein A or protein G or other similar Fc-binding matrices, and include the Fc regions of murine IgG, IgA, IgE, IgD, IgM or fragments of the human IgG IgA, IgE, IgD, IgM Fc region, *e.g.,* fragments comprising at least the hinge region so that interchain disulfide bonds will form.

IgG_1 Fc region is disclosed by, for example, GenBank Accession no. X70421, which is herein incorporated by reference in its entirety.

In one embodiment, the second amino acid sequence comprises the sequence set forth in SEQ ID NO:16.

In some embodiments, the second amino acid sequence preferably is C-terminus to the first amino acid sequence. Preparation of fusion polypeptides comprising a heterologous amino acid sequence fused to various portions of another amino acid sequence is described, *e.g.,* by Ashkenazi *et al.,* PNAS, 88:10535 (1991) and Byrn *et al.,* Nature 344:677 (1990), each of which is herein incorporated by reference in its entirety. For example, a gene fusion encoding the polypeptide comprising the first and the second amino acid sequences can be inserted into an appropriate expression vector. The expressed fusion proteins can be allowed to assemble, whereby interchain disulfide bonds can form between the polypeptides, yielding dimers. In other embodiments, the fusion polymers of the present invention can be expressed with or without spacer amino acid linking groups. For example, in some embodiments, the polypeptides of the present invention can further comprise a linker between the first and the second amino acid

sequence, wherein the linker comprises one or more amino acid residues separating the first and second sequences.

In another embodiment, the polypeptide of the present invention comprises the amino acid sequence set forth in SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:36, SEQ ID NO:38, or SEQ ID NO:39.

In one embodiment, the polypeptide is encoded by a nucleic acid molecule having the nucleotide sequence set forth in SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:37, SEQ ID NO:42, or SEQ ID NO:43.

Variants of the sequences disclosed herein also are within the scope of the present invention. A variant of a polypeptide may refer to an amino acid sequence that is altered by one or more amino acids. The variant can have "conservative" changes, wherein a substituted amino acid has similar structural or chemical properties, *e.g.*, replacement of leucine with isoleucine. Alternatively, a variant can have "non-conservative" changes, *e.g.*, replacement of a glycine with a tryptophan. Analogous minor variation can also include amino acid deletion or insertion, or both. A particular form of a "variant" polypeptide is a "functionally equivalent" polypeptide, *i.e.*, a polypeptide which exhibits substantially similar *in vivo* or *in vitro* activity and/or binding as the examples of the polypeptide of invention. Guidance in determining which amino acid residues can be substituted, inserted, or deleted without eliminating biological or immunological activity can be found using computer programs well-known in the art, for example, DNASTAR software (DNASTAR, Inc., Madison, WI). Further, specific guidance is provided below, including that provided within the cited references which are herein incorporated by reference.

In other embodiments, the specific positions of the named residues can vary somewhat while still being present in the polypeptide at structurally and functionally analogous positions (see Chang, Y., *et al.*, *Biochemistry 37*:3258-3271 (1998).

Further, particular embodiments of the invention can be characterized functionally relative to a vWF polypeptide, or a fragment thereof, in terms of FVIII binding ability. In some embodiments, a polypeptide of the invention exhibits a binding capacity towards a FVIII that is less than, about equal to, or greater than the binding capacity of a reference vWF protein (*e.g.*, a wild-type endogenous vWF), or a fragment thereof, that is capable of binding to a FVIII protein (*e.g.*, a wild-type endogenous FVIII).

Thus, the invention includes such variations of the polypeptides disclosed herein. Such variants include deletions, insertions, inversions, repeats, and substitutions. Further guidance concerning which amino acid changes are likely to be phenotypically silent can be found in Bowie, J. U., *et al.,* "Deciphering the Message in Protein Sequences: Tolerance to Amino Acid Substitutions," *Science 247*:1306-1310 (1990).

Thus, fragments, derivatives or analogs of the polypeptides of the present invention include fragments, derivatives or analogs having sequences that have, as compared to the polypeptides of the present invention, (i) one or more of the amino acid residues (*e.g.*, 1, 3, 5, 8, 10, 15 or 20 residues) substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue). Such substituted amino acid residues may or may not be one encoded by the genetic code; or (ii) one or more of the amino acid residues (*e.g.*, 1, 3, 5, 8, 10, 15 or 20 residues) include a substituent group. In other embodiments, fragments, derivatives or analogs of a polypeptide of the present invention include the polypeptide of the present invention that is coupled with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or that is one in which additional amino acids are fused to the polypeptide. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

As indicated, changes are preferably of a minor nature, such as conservative amino acid substitutions that do not significantly affect the folding or FVIII binding capacity. Of course, the number of amino acid substitutions a skilled artisan would make depends on many factors, including those described above. In some embodiments, the number of substitutions for any given polypeptide will not be more than 50, 40, 30, 25, 20, 15, 10, 5, 3, 2, or 1.

Amino acids residues (of the polypeptides of the present invention) that are essential for binding to FVIII can be identified by methods known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, *Science 244*:1081-1085 (1989)). The latter procedure introduces single alanine mutations at every residue in the molecule. The resulting mutant molecules are then tested for binding to FVIII, *e.g.*, as described herein. Sites that are critical for binding to FVIII can also be determined by structural analysis such as crystallization, nuclear magnetic resonance or photoaffinity labeling (Smith, *et al.*, *J. Mol. Biol. 224*:399-904 (1992) and de Vos, *et al. Science 255*:306-312 (1992)).

In one embodiment, the recombinant polypeptide has an amino acid sequences that is at least 70%, 80%, 90%, 95%, 98%, or greater identical to any one of the amino acid sequences set forth herein.

In another embodiment, the first amino acid sequence is present in a vWF polypeptide comprising the amino acid sequence set forth in SEQ ID NO:29 or a variant or fragment thereof.

In other embodiments, the first amino acid sequence is present in a vWF polypeptide encoded by a nucleic acid sequence set forth in SEQ ID NO:30 or a variant or fragment thereof.

In other aspects, the present invention provides a recombinant vWF-Fc fusion protein, wherein the vWF portion of the fusion protein is a truncated vWF that lacks at least one domain of a mature full-length vWF polypeptide, wherein the fusion protein is capable of forming multimers that are capable of binding a FVIII protein. In one embodiment, the truncated vWF has domains D' and D3, with the proviso that the truncated vWF lacks domain A1, A2, A3, D4, B1, B2, B3, C1, C2, CK, or a combination thereof. In another embodiment, the truncated vWF has domains D', D3, and A1, with the proviso that the truncated vWF lacks domains A2, A3, D4, B1, B2, B3, C1, C2, and CK. In some embodiments, the truncated vWF has domains D', D3, A1, and A2, with the proviso that the truncated vWF lacks domains A3, D4, B1, B2, B3, C1, C2, and CK. In other embodiments, the truncated vWF has domains D', D3, A1, A2, and A3, with the proviso that the truncated vWF lacks domains D4, B1, B2, B3, C1, C2, and CK. In still further embodiments, the truncated vWF lacks domains D4, B1, B2, B3, C1, C2, and CK.

III. Nucleic Acids, Vectors, and Expression systems

In other aspects, the present invention provides recombinant expression vectors for expression of the polypeptide comprising the first and the second amino acid sequences, and host cells transformed with the expression vectors. Any suitable expression system may be employed. The vectors comprise a first and a second DNA sequence encoding the first and the second amino acid sequences, respectively, operably linked to suitable transcriptional or translational regulatory nucleotide sequences, such as those derived from a mammalian, microbial, viral, or insect gene. Examples of regulatory sequences include transcriptional promoters, operators, or enhancers, an mRNA ribosomal binding site, and appropriate sequences which control transcription and translation initiation and termination. Nucleotide sequences are operably linked when the regulatory sequence functionally relates to the encoding DNA sequence. Thus, a promoter nucleotide sequence is operably linked to the encoding DNA sequence if the promoter

nucleotide sequence controls the transcription of the encoding DNA sequence. The ability to replicate in the desired host cells, usually conferred by an origin of replication, and a selection gene by which transformants are identified, may additionally be incorporated into the expression vector.

In still further embodiments, DNA sequences encoding appropriate signal peptides that may or may not be native to the first amino acid sequence can be incorporated into expression vectors. For example, a DNA sequence for a signal peptide (secretory leader) may be provided in frame to the first sequence so that the expressed polypeptide is initially translated as a fusion protein comprising the signal peptide. A signal peptide that is functional in the intended host cells enhances extracellular secretion of the polypeptide comprising the first and the second amino acid sequences. In some embodiments, the signal peptide is cleaved from the polypeptide upon secretion of the polypeptide from the cell. In other embodiments, appropriate signal peptides that are not native to the first amino acid sequence can be provided as an alternative to or in addition to a native signal sequence.

In some embodiments, the signal peptide has the amino acid sequence shown as SEQ ID NO:40.

Suitable host cells for expression of the polypeptides of the present invention include prokaryotes, yeast, filamentous fungi, or higher eukaryotic cells. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are described, for example, in Pouwels *et al.* Cloning Vectors: A Laboratory Manual, Elsevier, New York, (1985). Cell-free translation systems could also be employed to produce the polypeptides of the present invention using RNAs derived from DNA constructs.

Prokaryotes include gram negative or gram positive organisms, for example, *E. coli*. Suitable prokaryotic host cells for transformation include, for example, *E. coli*, *Bacillus subtilis*, *Salmonella typhimurium*, and various other species within the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*.

Expression vectors for use in prokaryotic host cells generally comprise one or more phenotypic selectable marker genes. A phenotypic selectable marker gene is, for example, a gene encoding a protein that confers antibiotic resistance or that supplies an autotrophic requirement. Among vectors preferred for use in bacteria include *e.g.*, pET24b or pET22b available from Novagen, Madison, WI (pET-24b(+) and pET-22b(+) = pET Expression System 24b (Cat. No.

69750) and 22b (Cat. No. 70765), respectively, EMD Biosciences, Inc., Novagen Brand, Madison, WI; see http://worldwideweb.emdbiosciences.com product information section regarding pET-24b and pET-22b for details regarding vector), pQE70, pQE60 and pQE-9, available from Qiagen Inc., Valencia, CA; pBS vectors, PHAGESCRIPT vectors, BLUESCRIPT vectors, pNH8A, pNH16a, pNH18A, pNH46A, available from Stratagene, LaJolla, CA; and ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia (now Pfizer, Inc., New York, NY). Among preferred eukaryotic vectors are pWLNEO, pSV2CAT, pOG44, pXT1 and pSG available from Stratagene; and pSVK3, pBPV, pMSG and pSVL available from Pharmacia. Other suitable vectors will be readily apparent to the skilled artisan.

Bacterial promoters suitable for use in the present invention include the *E. coli* lacI and lacZ promoters, the T3 and T7 promoters, the gpt promoter, the lambda PR and PL promoters, and the trp promoter. Suitable eukaryotic promoters include the CMV immediate early promoter, the HSV thymidine kinase promoter, the early and late SV40 promoters, the promoters of retroviral LTRs, such as those of the Rous sarcoma virus (RSV), and metallothionein promoters, such as the mouse metallothionein-I promoter. For example, promoter sequences used for recombinant prokaryotic host cell expression vectors include, but are not limited to, β-lactamase (penicillinase), lactose promoter, tryptophan (trp) promoter system, and tac promoter (Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1982). A particularly useful prokaryotic host cell expression system employs a phage λPL promoter and a cI857ts thermolabile repressor sequence. Plasmid vectors available from the American Type Culture Collection which incorporate derivatives of the λPL promoter include plasmid pHUB2 (resident in *E. coli* strain JMB9 (ATCC 37092)) and pPLc28 (resident in *E. coli* RR1 (ATCC 53082)).

The polypeptides of the present invention also may be expressed in yeast host cells, preferably from the *Saccharomyces* genus (*e.g., S. cerevisiae*). Other genera of yeast, such as *Pichia* or *Kluyveromyces*, may also be employed. Yeast vectors will often contain an origin of replication sequence from a 2μ yeast plasmid, an autonomously replicating sequence (ARS), a promoter region, sequences for polyadenylation, sequences for transcription termination, and a selectable marker gene. Suitable promoter sequences for yeast vectors include, among others, promoters for metallothionein, 3-phosphoglycerate kinase or other glycolytic enzymes, such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase,

phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Other suitable vectors and promoters for use in yeast expression include the glucose-repressible ADH2 promoter. Shuttle vectors replicable in both yeast and *E. coli* may be constructed by inserting DNA sequences from pBR322 for selection and replication in *E. coli* (Amp^r gene and origin of replication) into the yeast vectors.

In some embodiments, the yeast α-factor leader sequence may be employed to direct secretion of the polypeptide. The α-factor leader sequence can be inserted between the promoter sequence and the structural gene sequence. Other leader sequences suitable for facilitating secretion of recombinant polypeptides from yeast hosts are known to those of skill in the art. A leader sequence may be modified near its 3' end to contain one or more restriction sites. This will facilitate fusion of the leader sequence to the structural gene.

Yeast transformation protocols are known to those of skill in the art. One such protocol is described by Hinnen *et al.*, Proc. Natl. Acad. Sci. USA 75:1929, 1978. The Hinnen *et al.* protocol selects for Trp^+ transformants in a selective medium, wherein the selective medium consists of 0.67% yeast nitrogen base, 0.5% casamino acids, 2% glucose, 10 μ/ml adenine and 20 μg/ml uracil. Yeast host cells transformed by vectors containing ADH2 promoter sequence may be grown for inducing expression in a rich medium. An example of a rich medium is one consisting of 1% yeast extract, 2% peptone, and 1% glucose supplemented with 80 μg/ml adenine and 80 μg/ml uracil. Derepression of the ADH2 promoter occurs when glucose is exhausted from the medium.

Mammalian or insect host cell culture systems also can be employed to express recombinant polypeptides. Baculovirus systems for production of heterologous proteins in insect cells are reviewed by Luckow et al., Bio/Technology 6:47 (1988). Established cell lines of mammalian origin also may be employed. Examples of suitable mammalian host cell lines include the COS-7 line of monkey kidney cells (ATCC CRL 1651), L cells, C127 cells, 3T3 cells (ATCC CCL 163), Chinese hamster ovary (CHO) cells, HeLa cells, and BHK (ATCC CRL 10) cell lines, and the CV-1/EBNA-1 cell line derived from the African green monkey kidney cell line CVI (ATCC CCL 70) as described by McMahan *et al.*, EMBO J. 10:2821 (1991).

Other suitable cell lines include, but are not limited to, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS-1), human hepatocellular carcinoma cells (*e.g.*, Hep

G2), human adenovirus transformed 293 cells, mouse L-929 cells, HaK hamster cell lines, murine 3T3 cells derived from Swiss, Balb-c or NIH mice and a number of other cell lines. Another suitable mammalian cell line is the CV-1 cell line. Normal diploid cells, cell strains derived from in vitro culture of primary tissue, as well as primary explants, are also suitable. Candidate cells may be genotypically deficient in the selection gene, or may contain a dominantly acting selection gene.

In some embodiments, introduction of a vector construct into the cultured host cell can be effected by calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection or other methods. Such methods are described in many standard laboratory manuals, such as Davis *et al.*, Basic Methods In Molecular Biology, 2nd Edition (1995).

For example, the host cells can be transformed with the one or more vectors carrying the DNA comprising a nucleotide sequence encoding the polypeptide of the present invention, *e.g.* by methods known in the art, and can then be cultured under suitable conditions if desired, with amplification of one or both introduced genes. The expressed polypeptide can then be recovered and purified from the culture medium (or from the cell, for example if expressed intracellularly) by methods known to one of skill in the art. In some embodiments, the expressed polypeptide can be prepared as a protein complex, *e.g.* as a homodimer by virtue of one or more inter-chain disulfide bonds between two separate polypeptides with or without the FVIII.

Transcriptional and translational control sequences for mammalian host cell expression vectors may derive from viral genomes. Commonly used promoter sequences and enhancer sequences are derived from Polyoma virus, Adenovirus 2, Simian Virus 40 (SV40), and cytomegalovirus (CMV). DNA sequences derived from the SV40 viral genome, for example, SV40 origin, early and late promoter, enhancer, splice, and polyadenylation sites may be used to provide other genetic elements for expression of a structural gene sequence in a mammalian host cell. Viral early and late promoters are particularly useful because both are easily obtained from a viral genome as a fragment which may also contain a viral origin of replication.

Vectors suitable for replication in mammalian cells can include viral replicons, or sequences that ensure integration of the sequence encoding the polypeptide into the host genome. Suitable vectors can include, for example, those derived from simian virus SV40, retroviruses, bovine papilloma virus, vaccinia virus, and adenovirus. The components of the vectors, *e.g.*

replicons, selection genes, enhancers, promoters, and the like, may be obtained from natural sources or synthesized by known procedures.

A suitable vector, for example, can be one derived from vaccinia viruses. In this case, the heterologous DNA is inserted into the vaccinia genome. Techniques for the insertion of foreign DNA into the vaccinia virus genome are known in the art, and utilize, for example, homologous recombination. The insertion of the heterologous DNA is generally into a gene which is non-essential in nature, for example, the thymidine kinase gene (tk), which also provides a selectable marker.

Thus, mammalian expression vectors can comprise one or more eukaryotic transcription units that are capable of expression in mammalian cells. For example, the transcription unit can comprise at least a promoter element to mediate transcription of foreign DNA sequences. In some embodiments, promoters for mammalian cells include viral promoters such as that from SV40, CMV, Rous sarcoma virus (RSV), adenovirus (ADV), and bovine papilloma virus (BPV).

The transcription unit also can comprise a termination sequence and poly(A) addition sequences operably linked to the sequence encoding the polypeptide. The transcription unit also can comprise an enhancer sequence for increasing expression.

Optionally, sequences that allow for amplification of the gene also can be included, as can sequences encoding selectable markers. Selectable markers for mammalian cells are known in the art, and include for example, thymidine kinase, dihydrofolate reductase (together with methotrexate as a DHFR amplifier), aminoglycoside phosphotransferase, hygromycin B phosphotransferase, asparagine synthetase, adenosine deaminase, metallothionien, and antibiotic resistant genes such as neomycin. Or, for example, the vector DNA can comprise all or part of the bovine papilloma virus genome and be carried in cell lines such as C127 mouse cells as a stable episomal element.

Non-limiting examples of expression vectors and systems for use in mammalian host cells can be constructed, *e.g.*, as disclosed by Okayama *et al.*, Mol. Cell. Biol. 3:280 (1983), Cosman *et al.*, Mol. Immunol. 23:935 (1986) (system for stable high level expression of DNAs in C127 murine mammary epithelial cells), Cosman *et al.*, Nature 312:768 (1984) (expression vector PMLSV N1/N4; ATCC 39890), EP-A-0367566, and U.S. Pat. No. 5,350,683, each of which is herein incorporated by reference for its teaching of expression vectors and/or systems. Vectors may be derived from retroviruses. In some embodiments, in place of the native signal

sequence, a heterologous signal sequence may be included, such as the signal sequence for interleukin-7 (IL-7) described by, *e.g.*, U.S. Pat,. No. 4,965,195; the signal sequence for interleukin-2 receptor described by, *e.g.*, Cosman *et al.*, Nature 312:768 (1984); the interleukin-4 signal peptide described by, *e.g.*, EP 367,566; the type I interleukin-1 receptor signal peptide described by, *e.g.*, U.S. Pat. No. 4,968,607; and the type II interleukin-1 receptor signal peptide described by, *e.g.*, EP 460,846, each of which is incorporated herein by reference for its teaching of signal sequences.

In one embodiment, the recombinant polypeptide can be prepared using the PER.C6® technology (Crucell, Holland, The Netherlands). Expression of recombinant proteins is disclosed by, *e.g.*, U.S. Patent No. 6,855,544, which is herein incorporated by reference for its teaching of methods and compositions for the production of recombinant proteins in a human cell line.

It is also contemplated that the polypeptides of the present invention can be prepared by solid phase synthetic methods. See Houghten, R. A., *Proc. Natl. Acad. Sci. USA 82*:5131-5135 (1985); and U.S. Pat. No. 4,631,211 to Houghten *et al.* (1986).

In other embodiments, the present invention also encompasses recombinant polypeptides comprising the first and the second amino acid sequences, wherein the polypetides are differentially modified during or after translation, *e.g.*, by glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, *etc.* Any of numerous chemical modifications can be carried out by known techniques, including but not limited, to specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, *S. aureus* V8 protease, $NaBH_4$; acetylation, deamidation, formylation, methylation, oxidation, reduction; metabolic synthesis in the presence of tunicamycin; *etc.* Additional post-translational modifications encompassed by the invention include, for example, *e.g.*, N-linked or O-linked carbohydrate chains, processing of N-terminal or C-terminal ends, attachment of chemical moieties to the amino acid backbone, chemical modifications of N-linked or O-linked carbohydrate chains, and addition of an N-terminal methionine residue as a result of vectors and constructs adapted for expression of the recombinant polypeptides, for example for expression in prokaryotic cultured host cells.

In some embodiments, wherein insoluble polypeptides are isolated from a host cell (*e.g.* a prokaryotic host cell), the host cell can be exposed to a buffer of suitable ionic strength to solubilize most host proteins, but in which aggregated polypeptides of interest may be substantially insoluble, and disrupting the cells so as to release the inclusion bodies and make them available for recovery by, for example, centrifugation. This technique is known to one of ordinary skill in the art, and a variation is described, for example, in U.S. Pat. No. 4,511,503, which is incorporated by reference herein for its teaching of a method of solubilizing heterologous protein, produced in an insoluble refractile form in a recombinant host cell culture. Without being held to a particular theory, it is believed that expression of a recombinant protein, in *e.g. E. coli,* may result in the intracellular deposition of the recombinant protein in insoluble aggregates called inclusion bodies. Deposition of recombinant proteins in inclusion bodies can be advantageous both because the inclusion bodies accumulate highly purified recombinant protein and because protein sequestered in inclusion bodies is protected from the action of bacterial proteases.

Generally, host cells (*e.g., E. coli* cells) are harvested after an appropriate amount of growth and suspended in a suitable buffer prior to disruption by lysis using techniques such as, for example, mechanical methods (*e.g.,* sonic oscillator) or by chemical or enzymatic methods. Examples of chemical or enzymatic methods of cell disruption include spheroplasting, which comprises the use of lysozyme to lyse bacterial wall, and osmotic shock, which involves treatment of viable cells with a solution of high tonicity and with a cold-water wash of low tonicity to release the polypeptides.

Following host cell disruption, the suspension is typically centrifuged to pellet the inclusion bodies. The resulting pellet contains substantially all of the insoluble polypeptide fraction, but if the cell disruption process is not complete, it may also contain intact cells or broken cell fragments. Completeness of cell disruption can be assayed by resuspending the pellet in a small amount of the same buffer solution and examining the suspension with a phase-contrast microscope. The presence of broken cell fragments or whole cells indicates that additional disruption is necessary to remove the fragments or cells and the associated non-refractile polypeptides. After such further disruption, if required, the suspension can be again centrifuged and the pellet recovered, resuspended, and analyzed. The process can be repeated until visual examination reveals the absence of broken cell fragments in the pelleted material or

until further treatment fails to reduce the size of the resulting pellet. Once obtained from the solubilized inclusion bodies or at a later stage of purification, the polypeptide can be suitably refolded in a suitable refolding buffer such as those known in the art. The degree of any unfolding can be determined by chromatography including reversed phase-high performance liquid chromatography (RP-HPLC).

If the recombinantly expressed polypeptides of the present invention are not already in soluble form before they are to be refolded, they may be solubilized by incubation in a solubilization buffer comprising chaotropic agent (*e.g.,* urea, guanidine) and reducing agent (*e.g.,* glutathione, dithiothreitol (DTT), cysteine) in amounts necessary to substantially solubilize the polypeptides. This incubation takes place under conditions of polypeptide concentration, incubation time, and incubation temperature that will allow solubilization of the polypeptide to occur. Measurement of the degree of solubilization can be carried out by turbidity determination, by analyzing polypeptide fractionation between the supernatant and pellet after centrifugation on reduced SDS gels, by protein assay (*e.g.,* the Bio-Rad protein assay kit), or by high performance liquid chromatography (HPLC).

The pH of the solubilization buffer can be alkaline, preferably at least about pH 7.5, with the preferred range being about pH 7.5 to about pH 11. The concentration of the polypeptide of the present invention in the buffered solution for solubilization must be such that the polypeptide will be substantially solubilized and partially or fully reduced and denatured. Alternatively, the recombinant polypeptide may be initially insoluble. The exact amount to employ will depend, *e.g.,* on the concentrations and types of other ingredients in the buffered solution, particularly the type and amount of reducing agent, the type and amount of chaotropic agent, and the pH of the buffer. For example, the concentration of recombinant polypeptide can be increased if the concentration of reducing agent, *e.g.,* glutathione, is concurrently increased.

In still further embodiments, the present invention provides homogenous or substantially homogeneous polypeptides comprising the first and the second amino acid sequences. In one embodiment, the present invention provides an isolated polypeptide comprising the first amino acid sequence present in the vWF polypeptide and the second amino acid sequence heterologous to the first, wherein the polypeptide is capable of binding the FVIII. In other embodiments, the polypeptide is purified to substantial homogeneity, as indicated by a single protein band upon analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

As the skilled artisan will recognize, procedures for purifying recombinant proteins will vary according to such factors as the type of host cells employed and whether or not the proteins are secreted into the culture medium. For example, when expression systems that secrete the recombinant protein are employed, the culture medium first may be concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate can be applied to a purification matrix such as a gel filtration medium. Alternatively, an anion exchange resin can be employed, for example, a matrix or substrate having pendant diethylaminoethyl (DEAE) groups. The matrices can be acrylamide, agarose, dextran, cellulose or other types commonly employed in protein purification. Alternatively, a cation exchange step can be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Sulfopropyl groups are preferred. Finally, one or more reversed-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, (e.g., silica gel having pendant methyl or other aliphatic groups) can be employed to further purify the recombinantly expressed polypeptide. Some or all of the foregoing purification steps, in various combinations, can be employed to provide a substantially homogeneous recombinant protein.

In some embodiments, an affinity column comprising a binding partner of the structure or domain defined by the second amino acid sequence is employed to affinity-purify expressed recombinant polypeptides or protein complexes comprising them. For example, wherein the second amino acid sequence corresponds to an antibody Fc polypeptide, an affinity column comprising protein A or protein G can be used for affinity purification of the polypeptide or protein complexes comprising them. In some embodiments, bound polypeptides and/or complexes can be removed from an affinity column in a high salt elution buffer and then dialyzed into a lower salt buffer for use. By way of another example, the affinity column may comprise an antibody that binds the polypeptide or protein complexes comprising them, *e.g.,* an antibody against the structure or domain defined by the first or the second amino acid sequence.

In other aspects, a nucleotide sequence encoding the polypeptide of the present is provided, wherein the polypeptide comprises a first amino acid sequence present in a vWF polypeptide and a second amino acid sequence heterologous to the first, wherein the polypeptide is capable of binding a FVIII. In one embodiment, the present invention provides an isolated

nucleic acid molecule comprising the nucleotide sequence set forth in SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, 37, 42, and 43.

The polynucleotides of the invention can include variants which have substitutions, deletions, and/or additions which can involve one or more nucleotides. The variants can be altered in coding regions, non-coding regions, or both. Alterations in the coding regions can produce conservative or non-conservative amino acid substitutions, deletions or additions. Especially preferred among these are silent substitutions, additions and deletions, which do not alter the properties and FVIII binding ability of the polypeptides of the present invention.

Further embodiments of the invention include nucleic acid molecules comprising a polynucleotide having a nucleotide sequence at least 90% identical, and more preferably at least 95%, 96%, 97%, 98% or 99% identical to (a) a nucleotide sequence encoding a polypeptide having the amino acid sequences set for herein; and (b) a nucleotide sequence complementary to any of the nucleotide sequences in (a) above.

By a polynucleotide having a nucleotide sequence at least, for example, 95% "identical" to a reference nucleotide sequence encoding a polypeptide is intended that the nucleotide sequence of the polynucleotide be identical to the reference sequence except that the polynucleotide sequence can include up to five point mutations per each 100 nucleotides of the reference nucleotide sequence encoding the polypeptide. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence can be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence can be inserted into the reference sequence. These mutations of the reference sequence can occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence.

Two or more polynucleotide sequences can be compared by determining their percent identity. Two or more amino acid sequences likewise can be compared by determining their percent identity. The percent identity of two sequences, whether nucleic acid or peptide sequences, is generally described as the number of exact matches between two aligned sequences divided by the length of the shorter sequence and multiplied by 100. An approximate alignment for nucleic acid sequences is provided by the local homology algorithm of Smith and Waterman,

Advances in Applied Mathematics 2:482-489 (1981). This algorithm can be extended to use with peptide sequences using the scoring matrix developed by Dayhoff, Atlas of Protein Sequences and Structure, M. O. Dayhoff ed., 5 suppl. 3:353-358, National Biomedical Research Foundation, Washington, D.C., USA, and normalized by Gribskov, *Nucl. Acids Res. 14(6)*:6745-6763 (1986). An implementation of this algorithm for nucleic acid and peptide sequences is provided by the Genetics Computer Group (Madison, Wis.) in their BESTFIT utility application. The default parameters for this method are described in the Wisconsin Sequence Analysis Package Program Manual, Version 8 (1995) (available from Genetics Computer Group, Madison, Wis.).

For example, due to the degeneracy of the genetic code, one of ordinary skill in the art will recognize that a number of the nucleic acid molecules having a sequence at least 90%, 95%, 96%, 97%, 98%, or 99% identical to any one of the nucleic acid sequences described herein can encode the polypeptide.

In fact, because degenerate variants of these nucleotide sequences all encode the same polypeptide, this will be clear to the skilled artisan even without performing any functional assays or measurements described herein. It will be further recognized in the art that, for such nucleic acid molecules that are not degenerate variants, a reasonable number will also encode a polypeptide having FVIII binding capability. This is because the skilled artisan is fully aware of amino acid substitutions that are either less likely or not likely to significantly effect protein binding (*e.g.*, replacing one aliphatic amino acid with a second aliphatic amino acid).

Recently, advances in the synthetic production of longer polynucleotide sequences have enabled the synthetic production of nucleic acids encoding significantly longer polypeptides without the use of traditional cloning techniques. Commercial providers of such services include Blue Heron, Inc., Bothell, WA (http://worldwideweb.blueheronbio.com). Technology utilized by Blue Heron, Inc. is described in U.S. Patent Nos. 6,664,112; 6,623,928; 6,613,508; 6,444,422; 6,312,893; 4,652,639; U.S. Published Patent Application Nos. 20020119456A1; 20020077471A1; and Published International Patent Applications (Publications Nos) WO03054232A3; WO0194366A1; WO9727331A2; and WO9905322A1, all incorporated herein by reference.

Of course, traditional techniques of molecular biology, microbiology, and recombinant nucleic acid can also be used to produce the polynucleotides of the invention. These techniques

are well known and are explained in, for example, Current Protocols in Molecular Biology, F. M. Ausebel, ed., Vols. I, II and III (1997); Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989); DNA Cloning: A Practical Approach, D. N. Glover, ed., Vols. I and II (1985); Oligonucleotide Synthesis, M. L. Gait, ed. (1984); Nucleic Acid Hybridization, Hames and Higgins, eds. (1985); Transcription and Translation, Hames and Higgins, eds. (1984); Animal Cell Culture, R. I. Freshney, ed. (1986); Immobilized Cells and Enzymes, IRL Press (1986); Perbal, "A Practical Guide to Molecular Cloning"; the series, Methods in Enzymology, Academic Press, Inc. (1984); Gene Transfer Vectors for Mammalian Cells, J. H. Miller and M. P. Calos, eds., Cold Spring Harbor Laboratory (1987); and Methods in Enzymology, Wu and Grossman and Wu, eds., respectively, Vols. 154 and 155, all incorporated herein by reference.

Also provided, in other aspects, is an expression vector comprising the nucleic acid molecules encoding the polypeptides of the present invention. Host cells also are provided that express the polypeptides of the present invention. In one embodiment, the present invention provides a cell expressing a polypeptide comprising a first amino acid sequence present in a vWF polypeptide and a second amino acid sequence heterologous to the first, wherein the polypeptide is capable of binding a FVIII, wherein the cell further expresses the FVIII. In another embodiment, the FVIII is recombinant FVIII.

IV. Protein Complex

In another aspect, the present invention provides a protein complex comprising a polypeptide and a FVIII, wherein the polypeptide comprises a first amino acid sequence present in a vWF polypeptide and a second amino acid sequence heterologous to the first, wherein the polypeptide is capable of binding the FVIII. In one embodiment, the complex comprises two separate chains of the polypeptide in the form of a dimer in complex with the FVIII.

In another embodiment, the present invention provides for a homodimeric protein complex comprising two of the polypeptide chains of the present invention, wherein one or more disulfide bonds are formed between the chains. In one embodiment, one or more disulfide bonds form between the first amino acid sequences of two separate chains thereby creating a dimer. In another embodiments, one or more disulfide bonds form between Fc regions of two separate chains thereby creating a dimer. In some embodiment, the homodimeric complex consists of or

essentially consists of two of the polypeptide chains of the present invention. In still further embodiments, heterodimers also are within the scope of the present invention.

In another embodiment, the present invention provides oligomers, *e.g.* by further linking of the dimers. In some embodiments, differently sized oligomers are provided, preferably with formation of disulfide bridges at the amino-terminal ends of the polypeptides of the present invention. Thus, in other embodiments, differently sized oligomers ranging in size from a dimer of at least about: 100,000, 250,000, 500,000 daltons or more including large multimers of about: 5, 10, 20, 30, 40, or 50 million daltons or more are provided.

In still further embodiments, the oligomers are homo- or hetero-oligomers. In another embodiment, the dimer is a heterodimer.

In other embodiments, the protein complex is prepared from a cell or tissue culture expression system that expresses the polypeptide and the FVIII. In one embodiment, the polypeptide and the FVIII are co-expressed in the same cell.

In one embodiment, the present invention provides a soluble fusion protein comprising a first amino acid sequence fused to the N-terminus of an Fc polypeptide, wherein the first amino acid sequence is present in a vWF polypeptide, wherein the polypeptide is capable of binding a FVIII. In some embodiments, the polypeptide is capable of binding the FVIII as a dimer comprising two of the soluble fusion proteins joined by disulfide bonds.

In another embodiment, the present invention provides a dimer comprising two soluble fusion proteins joined by disulfide bonds, wherein each protein comprises a first amino acid sequence fused to the N-terminus of an Fc polypeptide, wherein the first amino acid sequence is present in a vWF polypeptide, wherein the dimer is capable of binding a FVIII.

In other aspects, the present invention provides a protein complex comprising disulfide-linked multimers comprising two or more of the polypeptide having the first and the second amino acid sequence.

In one embodiment, the disulfide-linked multimers are prepared by contacting the polypeptides with a vWF propeptide fragment whereby the vWF propeptide fragment acts in "*trans*" to direct the assembly of the disulfide-linked multimers.

In some embodiment, the vWF propeptide fragment comprises the nucleotide sequence set forth in SEQ ID NO:31 or a variant thereof.

In another embodiment, the contacting comprises co-expressing the polypeptide with the vWF propeptide fragment.

For example, in one embodiment, the present invention provides a protein complex comprising disulfide-linked multimers comprising two or more of the polypeptide having the first and the second amino acid sequence, wherein the first amino acid sequence is set forth in SEQ ID NO:1, SEQ ID NO:4, or SEQ ID NO:7. In other embodiments, the first amino acid sequence is set forth in SEQ ID NO:3, SEQ ID NO:6, or SEQ ID NO:9. In some embodiments, the first amino acid sequence is set forth in SEQ ID NO:17, SEQ ID NO:18, or SEQ ID NO:19. In another embodiment, the protein complex is prepared by co-expressing, using a recombinant expression system, the polypeptide with the vWF propeptide fragment comprising the amino acid sequence set forth in SEQ ID NO:31 whereby the fragment acts in "*trans*" to direct the assembly of disulfide-linked multimers comprising the polypeptides.

V. Methods

In still further aspects, the present invention provides a method for preparing a protein complex of the present invention. In some embodiments, an affinity column comprising a binding partner of the structure or domain defined by the second amino acid sequence is employed to affinity-purify the complex. For example, wherein the second amino acid sequence corresponds to an antibody Fc polypeptide, an affinity column comprising protein A or protein G can be used for affinity purification of the complex. In some embodiments, the binding partner is immobilized. Alternatively, the affinity column may comprise an antibody that binds the Fc portion of the polypeptide or that binds a structure or domain defined by the first amino acid sequence of the polypeptide. In some embodiments, the complex to be prepared comprises a dimer comprising two soluble fusion proteins joined by disulfide bonds, wherein each protein comprises a first amino acid sequence fused to the N-terminus of an Fc polypeptide, wherein the first amino acid sequence is present in a vWF polypeptide, wherein the dimer is capable of binding a FVIII.

In another embodiment, the complex further comprises FVIII bound to the dimer. FVIII can be removed/dissociated from the complex and, optionally, subjected to one or more additional purification steps to obtain partially pure, substantially pure, or pure FVIII.

In one aspect, the present invention provides a method for preparing a FVIII, the method comprising: contacting the FVIII with a polypeptide to form a protein complex comprising the FVIII and the polypeptide, wherein the polypeptide comprises a first amino acid sequence present in a vWF polypeptide and a second amino acid sequence heterologous to the first, wherein the polypeptide is capable of binding the FVIII to form the protein complex.

In one embodiment, the method further comprises selectively adhering the complex to a separation medium comprising a binding partner having affinity for a region or domain defined by the second amino acid sequence. In another embodiment, the second amino acid sequence corresponds to an immunoglobulin Fc region. In other embodiments, the binding partner is a protein A or a protein G. In one embodiment, the binding partner is an antibody. In some embodiments, the binding partner is an antibody against the immunoglobulin Fc region. In still further embodiments, the complex comprises two chains of the polypeptide in the form of a dimer, wherein the dimer is affinity-bound to the FVIII.

For immobilization of the binding partner, any number of different solid supports may be utilized. For example, the solid support material may be composed of polysaccharides, such as cellulose, starch, dextran, agar or agarose, or hydrophilic synthetic polymers, such as substituted or unsubstituted polyacrylamides, polymethacrylamides, polyacrylates, polymethacrylates, polyvinyl hydrophilic polymers, polystyrene, polysulfone or the like. Other suitable materials for use as the solid support material include porous mineral materials, such as silica, alumina, titania oxide, zirconia oxide and other ceramic structures. Alternatively, composite materials may be used as the solid support material. Such composite materials may be formed by the copolymerization of or by an interpenetrated network of two or more of the above-mentioned entities. Examples of suitable composite materials include polysaccharide-synthetic polymers and/or polysaccharide-mineral structures and/or synthetic polymer-mineral structures, such as are disclosed in U.S. Pat. Nos. 5,268,097, 5,234,991, and 5,075,371, each of which is herein incorporated by reference for its teaching of composite materials.

The solid support material of the present invention may take the form of beads or irregular particles ranging in size from about 0.1 mm to 1000 mm in diameter, fibers (hollow or otherwise) of any size, membranes, flat surfaces ranging in thickness from about 0.1 mm to 1 mm thick, and sponge-like materials with holes from a μm to several mm in diameter.

Preferably, the binding partners are chemically immobilized on the solid support material via a covalent bond formed between, *e.g.,* a mercapto group of the binding partner and a reactive group present on the solid support. Reactive groups capable of reacting with the mercapto group of the present ligand include epoxy groups, tosylates, tresylates, halides and vinyl groups. Because many of the aforementioned solid support materials do not include one of the reactive groups recited above, bifunctional activating agents capable of both reacting with the solid support materials and providing the necessary reactive groups may be used. Examples of suitable activating agents include epichlorhydrin, epibromhydrin, dibromo- and dichloropropanol, dibromobutane, ethyleneglycol diglycidylether, butanediol diglycidylether, divinyl sulfone and the like.

Typical examples of suitable supports are Sepharose™, agarose, the resin activated-CH Sepharose™ 4B (N-hydroxysuccinimide containing agarose) from Pharmacia (Sweden), the resin NHS-activated Sepharose™ 4 Fast Flow (activated with 6-aminohexanoic acid to form active N-hydroxysuccinimide esters; Amersham Biosciences), the resin CNBr-activated Sepahrose™ Fast Flow (activated with cyanogen bromide; Amersham Biosciences) the resin PROTEIN PAK™ epoxy-activated affinity resin (Waters, USA), the resin EUPERGIT™ C30 N (Rohm & Haas, Germany), UltraLink Biosupport Medium (Pierce), Trisacryl GF-2000 (Pierce), or AFFI-GEL™ from BioRad (USA). Preferably, the support for affinity chromatography is preactivated with epoxyde groups for direct coupling to peptides and proteins.

The affinity chromatography resins useful for practicing the methods of the invention include, but are not limited to, any combination of ligand or compound described above with any of the supports described above. Non-limiting examples of specific affinity chromatography resins are Protein A-Sepharose™, Protein A-agarose, Protein A-agarose CL-4B, Protein G-Sepharose™, Protein G-agarose, Protein G-agarose CL-4B, Protein A/G agarose (various versions of all of the above are available from various manufacturers, *e.g.,* Sigma-Aldrich, Amersham, and Pierce), Protein A Ultraflow™ (Sterogene), Protein A Cellthru™ 300 (Sterogene), QuickMab (Sterogene), QuickProtein A™ (Sterogene), Thruput™ and Thruput Plus (Sterogene), PROSEP-A or PROSEP-G (Millipore), and any variations of the above.

The methods used for the affinity chromatography can depend, at least in part, on the specific reagent used and are typically supplied by the manufacturer or known in the art. For example, the affinity chromatography reagent can be packed in a chromatographic column,

equilibrated with a buffer capable of promoting an interaction between the protein complex and the binding partner, and then contacted with a composition comprising the complex. The column can then be washed with at least one solution capable of eluting the impurities without interfering with the interaction between the complex and the affinity ligand. The entire complex comprising the polypeptide and the FVIII can then be eluted using an appropriate eluent to obtain the complex. Alternatively, the FVIII can be dissociated from the complex whereby the complex remains bound to the binding partner.

For example, in a method for preparing a protein complex comprising the polypeptide (*e.g.*, a dimer of the polypeptide) with or without the FVIII bound thereto, a composition comprising the complex can be contacted with at least one affinity chromatography resin that has Protein A as a ligand under conditions that allow binding of the complex to the resin. Desirably, the Protein A is a naturally occurring or a recombinant form of Protein A. The chromatography resin can then be washed with a series of wash buffers having increasing acidity (*e.g.* pH 7.0, 6.5, 6.0, 5.8, 5.5, 5.2, 5.0, 4.8, 4.6, 4.5, 4.4, or 4.0) such that the washing causes the dissociation of non-complex material from the resin but does not substantially dissociate the complex. The resin can be washed at least one time, preferably at least two times, and most preferably at least three times with wash buffers, where the first wash buffer has a pH of about 5.0 to 6.0, preferably about 5.2, and each subsequent wash buffer has a pH that is more acidic than the previous wash buffer. In preferred embodiments, the wash buffers will not dissociate more than 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10%, or 5% of the complex from the resin.

In some embodiments, after washing the resin, the complex can be eluted from the chromatography resin using an eluent having an acidic pH of about 2.5 to 3.5 (*e.g.*, pH 2.5, 3.0, 3.5) and being more acidic than any of the wash buffers. The eluate contains the purified, partially purified, or substantially purified complex with a preferred purity of at least 50%, 60%, 70%, 80%, 85%, 90%, or 95% or more.

In other embodiments, wherein the protein complex to be prepared comprises the FVIII bound to the dimeric form of the polypeptide, resin binding and washing conditions can be performed under conditions such that the FVIII remains affinity-bound to the dimer, which in turn can remain bound to the resin. Then, the FVIII can be separated from the resin-bound dimer to provide a composition comprising the FVIII, wherein the composition is partially, substantially, or completely free of the dimer.

Thus, in some embodiments, the polypeptide comprising the first and the second amino acid sequences can be employed for preparing FVIII by virtue of its ability to bind the FVIII to form a protein complex comprising the polypeptide and the FVIII. In some embodiments, such a protein complex can be subjected to affinity chromatography to prepare the complex and/or any FVIII associated therewith.

In other aspects, the present invention provides a composition comprising the FVIII, wherein the FVIII is prepared according to the methods described herein. Also provided are compositions comprising a protein complex comprising the polypeptide having the first and the second amino acid sequence, with or without FVIII bound to the polypeptide of the complex. In one embodiment, the composition comprises a protein complex comprising two of the polypeptides in the form of a dimer (*e.g.,* homo- or hetero-dimer) bound with the FVIII.

Thus, in some aspects, the present invention provides compositions (*e.g.* truncated recombinant vWF fusion protein) and methods for enhancing expression of recombinant FVIII by mammalian cells by protecting it from removal and/or degradation, as well as to allow rapid chromatographic purification of the resulting FVIII:recombinant vWF complex by use of the fusion protein handle (*e.g.,* the second amino acid sequence) incorporated into the recombinant vWF polypeptide of the present invention.

Accordingly, in various embodiments, a robust and simple method is provided for using a truncated version of vWF coupled to the Fc portion of immunoglobulin (e.g., immunoglobulin G_1) to complex with, and protect, FVIII during expression and production in a manner analogous to a complete vWF molecule. The unique approach described herein is more efficient at least because of the smaller size and easier expression of the vWF fusion polypeptides of the present invention, and/or (2) the ability to rapidly purify the FVIII:vWF fusion complex through the Fc region on the fusion protein, thus selectively enriching for the desired FVIII molecule with less contamination from other proteins as well as from vWF. In addition, the high-affinity binding of an Fc region provides that a FVIII:vWF fusion complex can also be removed *in situ* through an inline protein A cartridge, for example, in order to increase the yield and purity.

In one embodiment, the present invention provides a method using in-line purification during a perfusion process to continually purify the FVIII:recombinant vWF fusion complex

through the selective affinity attachment of the vWF fusion Fc region to a protein A (or other similar) matrix.

The present invention, in some embodiments, takes advantage of at least two observations to create a superior method for FVIII purification: (a) using recombinant vWF polypeptides, in particular recombinant truncated vWF polypeptides that retain binding to FVIII but are significantly smaller than the full-length molecule, and (b) creating a fusion protein with the Fc region of an immunoglobulin (*e.g.*, IgG_1) that binds with high affinity to the ligand, Protein A. This combination provides robust expression in mammalian cell systems while allowing a rapid method for collecting the FVIII:recvWF complex without regard to media or other components in the expression supernatant, as well as issues related to ionic strength, etc. It furthers allows a variety of reagents/solutions to be used to remove the FVIII while retaining full activity, and with superior recovery, of product. The high-affinity binding of the Fc portion to Protein A (or high-affinity antibody) column does not interfere with the FVIII:vWF interaction, as it is a separate and independent molecular entity.

In still further aspects, the present invention provides a method for recombinantly preparing the polypeptides of the present invention. In some embodiments, the method comprises: (a) generating a mammalian cell line by transforming the cell line with an expression vector encoding the polypeptide of the present invention; (b) growing the cell line under conditions sufficient for expressing the polypeptide; and (c) purifying the expressed protein from step (b) to obtain a vWF-Fc fusion protein, wherein the vWF portion of the fusion protein is a truncated vWF that lacks at least one domain of a mature full-length vWF polypeptide, wherein the fusion protein is capable of forming multimers that are capable of binding a FVIII protein. In one embodiment, the truncated vWF has domains D' and D3, with the proviso that the truncated vWF lacks domain A1, A2, A3, D4, B, C1, C2, CK, or a combination thereof. In another embodiment, the truncated vWF has domains D', D3, and A1, with the proviso that the truncated vWF lacks domains A2, A3, D4, B, C1, C2, and CK. In some embodiments, the truncated vWF has domains D', D3, A1, and A2, with the proviso that the truncated vWF lacks domains A3, D4, B, C1, C2, and CK. In other embodiments, the truncated vWF has domains D', D3, A1, A2, and A3, with the proviso that the truncated vWF lacks domains D4, B, C1, C2, and CK. In still further embodiments, the truncated vWF lacks domains D4, B, C1, C2, and CK.In other embodiments, the method further comprises co-expressing a recombinant propeptide, wherein

the vWF-Fc fusion protein is expressed as a recombinant fusion protein that further lacks a propeptide sequence, wherein the recombinant propeptide and the recombinant vWF-Fc fusion associate to form a propeptide/vWF-Fc complex following recombinant expression.

VI. Other Compositions and Methods

In other aspects, the present invention provides methods and compositions for FVIII having extended plasma half-life. For example, in some embodiments, the recombinant vWF-Fc fusion polypeptides of the present invention can be employed as an additive to a recombinant or a plasma-derived FVIII that may promote an extended half-life for the FVIII and/or combined complex. In accordance with the present invention, in some embodiments, the Fc region is fused to a truncated piece of vWF such that FVIII binding is provided and the Fc region further provides for additional half-life to the complex. For example, in one embodiment, the recombinant FVIII:recombinant vWF-Fc fusion complex can be either purified directly from expressed culture media, then injected (*e.g.,* intravenously) into patients as a pharmaceutical formulation, e.g. with or without an excess of the recombinant vWF-Fc fusion (*i.e.,* the polypeptide of the present invention). In some embodiments, supplementing the complex with excess vWF-Fc fusion (*e.g.,* 2, 3, 4, 5, 6, 7, 8, 9, 10 fold or more on a mole basis) is provided in order to increase the fusion polypeptide concentration in plasma and provide for FVIII to re-bind to the vWF-Fc fusion protein, if it dissociates in the plasma.

In still further embodiments, the fusion polypeptides of the present invention can be formulated at high concentration with FVIII, administered to a human or non-human subject intravenously or by other routes thereby allowing the FVIII to be associated with vWF fusion protein during the course of its circulation and prior to cleavage by thrombin, released at the site of injury.

Accordingly, in some embodiments, the polypeptides of the present invention and/or protein complex comprising them, can be formulated for treatment. For example, the polypeptides and/or protein complexes of the present invention can be formulated according to known methods to prepare pharmaceutically useful compositions, preferably combined in admixture with a pharmaceutically acceptable carrier vehicle. Suitable vehicles and their formulation, inclusive of other human proteins, *e.g.* human serum albumin, are described for example in Remington's Pharmaceutical Sciences by E. W. Hartin, which is herein incorporated by reference. In one embodiment, such compositions will contain an effective amount of the

FVIII protein in complex with a recombinant vWF polypeptide, together with a suitable amount of vehicle in order to prepare pharmaceutically acceptable compositions suitable for effective administration to a subject, for example parenterally administered to a subject suffering, e.g., from hemophilia A.

The average current dosage for the treatment of a hemophiliac varies with the severity of the bleeding episode. For example, the average doses administered intravenously may be in the range of: about 40 units per kilogram of FVIII for pre-operative indications, about 15 to about 20 units per kilogram for minor hemorrhaging, and about 20 to about 40 units per kilogram administered over an about 8-hour period for a maintenance dose. Other dosages and regimens can be readily determined by one of ordinary skill in the art of treating hemophiliacs.

VII. Kit

In still further aspects, kits comprising the polypeptides, nucleic acid sequences, protein complexes, and/or compositions of the present invention also are provided. The kits can have a single container, or they may have distinct container for each desired component. Kits comprising reagents necessary for preparing the recombinant polypeptides and/or the protein complexes derived therefrom also are contemplated, for example reagents such as, but not limited to, expression vectors, recombinant host cells comprising the expression vectors, and purification reagents. Further, wherein the components of the kit are provided in one or more liquid solutions, the liquid solution is an aqueous solution, with a sterile aqueous solution being particularly preferred. However, the components of the kit may be provided as dried powder(s). When reagents or components are provided as a dry powder, the powder can be reconstituted by the addition of a suitable solvent. It is envisioned that the solvent may also be provided.

The following examples are given only to illustrate the present process and are not given to limit the invention. One skilled in the art will appreciate that the examples given only illustrate that which is claimed and that the present invention is only limited in scope by the appended claims.

EXAMPLES

Example 1
Construction of expression plasmids for truncated vWF-Fc fusion polypeptides

Six DNA molecules, each having a nucleotide sequence that encodes a truncated vWF-Fc fusion polypeptide, were commercially synthesized (GENEART AG, Regensburg, Germany). The sequence identifiers for the nucleotide sequences of the DNA molecules and for the corresponding encoded amino acid sequences are shown in Table 1.

Table 1: Nucleotide and amino acid sequences for truncated vWF-Fc polypeptides.

Polypeptide Name	Nucleotide Sequence	Amino Acid Sequence
D'-D3-Fc	SEQ ID NO:37	SEQ ID NO:36
Pro-D'-D3-Fc	SEQ ID NO:24	SEQ ID NO:20
D'-A1-Fc	SEQ ID NO:42	SEQ ID NO:38
Pro-D'-A1-Fc	SEQ ID NO:28	SEQ ID NO:21
D'-A3-Fc	SEQ ID NO:43	SEQ ID NO:39
Pro-D'-A3-Fc	SEQ ID NO:23	SEQ ID NO:22

For each DNA molecule, the nucleotide sequence portion of the molecule encoding the truncated vWF region was codon-optimized using algorithms that account for codon usage, secondary structure, inhibitory sequences, and the like. The Fc DNA region of each molecule, which corresponds to the amino acid sequence shown in SEQ ID NO:16 and which is derived from IgG_1, was not subjected to sequence-optimization. Further, each DNA molecule contains a nucleotide sequence that encodes the signal peptide sequence shown as SEQ ID NO:40. SEQ ID NOs:20-22 additionally include the propeptide amino acid sequence shown as SEQ ID NO:41.

Restriction sites included in the synthetic genes were cleaved and re-cloned into the corresponding restriction sites in the plasmid expression vector pcDNA3002Neo (Crucell, Netherlands). Recombinant DNAs were prepared from clones by the SDS-alkaline lysis method according to commonly known protocols, *e.g.* as described by Sambrook *et al.*, (1989) Molecular Cloning: A Laboratory Manual, CSH; Jones (1995) Gel Electrophoresis: Nucleic Acids Essential Techniques, Wiley. Restriction enzyme-digested DNAs were fractionated on 1% agarose gels to determine size and identity. Verification of plasmid clone sequences was performed by automated DNA sequencing using fluorescent deoxyribonucleotide primers (Applied Biosystems, Carlsbad, CA). The Prepared plasmid expression vector DNAs encoding the various truncated vWF-Fc constructs were each sterilized by a 70% ethanol wash after precipitation, and

resuspended in sterile water to a final concentration of about 0.2 to about1.0 micrograms per microliter.

Example 2
Electroporation of expression plasmids into PER.C6 Mammalian Cells

PER.C6 cells and cells of clonal line 078 (BDD-078 cells), which are PER.C6 cells expressing a B-domain-deleted FVIII (BDD-FVIII), were each maintained in continuous culture by routine (4 day) passaging in PER-MAb media (Hyclone, Logan, UT). Forty-eight hours prior to electroporation, cells were refreshed by seeding cells at 1 x 10^6 cells/ml in fresh PER-MAb medium. On the day of the electroporation, 5 x 10^6 cells were resuspended into 100 microliters of Amaxa Nucleofector® Kit V Solution (Lonza Walkersville Inc., Walkersville, MD).

For each electroporation, the cell suspension was mixed with about 2 to about 5 micrograms of expression plasmid DNA for expression of the polypeptides described in Table 1. The cell mixture was then transferred to the electroporation cuvette of an Amaxa Nucleofector® Device (Lonza Walkersville Inc., Walkersville, MD); and a pre-set program X-001 was used to electroporate the DNA into cells. After electroporation, the cell suspension was transferred immediately into pre-warmed (37°C) Mab media (SAFC Biosciences, Lenexa, Kansas) in the well of a 6-well plastic microplate. Cells were incubated without shaking in a humidified incubator chamber set at 37°C, with 5% CO_2 and 95% humidity.

After approximately 48 hours in culture, each electroporation reaction was removed from the 6-well microplate into a 125 culture flask with 20 ml of fresh MAb media (SAFC Bioscience, Lenexa, Kansas) containing appropriate selection antibiotic(s): Geneticin/G418 or Zeocin were used at final concentrations 125 or 100 micrograms/ml, respectively; Hygromycin was used at a final concentration of 50 micrograms/ml). Cells were then cultured at 37°C, in 5% CO_2 and 95% humidity without shaking. After 7-10 days, cells were then adapted to growth in PER-MAb media with shaking and routine passaging to prepare a polyclonal pool of transfected cells. Alternatively, selected cells were prepared for limited dilution cloning as described by, *e.g.*, Harlow, E and Lane, D. (1988). Antibodies: A Laboratory Manual, pp. 116-117 and pp. 222-223. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. Cloned cells were checked regularly for growth and eventually were moved into shake flasks for expansion and growth as suspension cells in PER-MAb medium (Hyclone, Logan, UT).

Example 3
Assays

FVIII: A commercial BDD-FVIII (a/k/a Xyntha®; Antihemophilic Factor (Recombinant)) (Wyeth (now a subsidiary of Pfizer Inc., New York, NY)) protein was used as the standard for developing a robust linear assay using an enzyme-linked immunosorbent assay (ELISA). The capture antibody bound to the microplate wells was a commercial murine monoclonal antibody, namely clone GMA-012 (R8B12) (Green Mountain Antibodies, Inc., Burlington, VT), directed against the A2 domain of human FVIII; and the detection antibody was a commercial biotinylated sheep polyclonal antibody, namely SAF8C-APBIO, (Affinity Biologicals, Ontario, Canada) that recognizes human FVIII. Washing of microwells between antibody additions was done in the presence of Tris-buffered saline (TBS), pH 7.5. Colorimetric detection at 405 nm was by streptavidin-alkaline phosphatase mediated-cleavage of pNpp substrate. Alternatively, colorimetric FVIII unit activity determination was performed using a Beckman ACL Coagulation Analyzer, performed according to the manufacturer's specifications and using an internal plasma calibrator control, or by the Chromogenix Coatest SP4 FVIII kit (Diapharma Group, Columbus, OH) using a BDD-FVIII standard.

vWF: Research-grade plasma-derived vWF (Haemtech, Essex Junction, VT) was used as an antigen standard for ELISA measurements of protein content. The capture antibody used is a commercial murine monoclonal against human vWF protein; and for detection, an HRP-conjugated goat anti-human vWF antibody was used. Colorimetric detection at 450 nm is by incubation with the enzyme substrate, tetramethylbenzidine.

For the recombinant vWF-Fc fusion proteins, similar ELISA formats were used, such as capture of the Fc region by protein A or anti-human Fc antibody immobilized on a microplate, followed by detection with murine anti-human vWF polyclonal antibodies binding to the D'-D3, D'-A1 or D'-A3 domains of vWF, depending on the expression construct.

Example 3
Characterization of truncated vWF-Fc polypeptides

The recombinant polypeptides corresponding to the truncated vWF-Fc proteins (*i.e.*, D'-D3-Fc, Pro-D'-D3-Fc, D'-A1-Fc, Pro-D'-A1-Fc, D'-A3-Fc, Pro-D'-A3-Fc; Table 1) that were

expressed in PER.C6 cells were immunoprecipitated by incubation of 0.2-1 ml of cell supernatant with 20 microliters of protein G beads. Beads were concentrated by centrifugation, then washed with Tris buffer. Centrifuged beads were suspended in 25 microliters of Laemmli buffer and heated to 95°C for 10 minutes before loading onto a 4-12% gradient Bis-Tris PAGE gel. Visualization of bands was by Coomassie Brilliant Blue staining/destaining.

For experiments involving either co-expression of BDD-FVIII and pro-D'-A3-Fc fusion or by mixing of commercial BDD-FVIII, Xyntha® (Wyeth), with PER.C6 cell supernatants expressing pro-D'-D3-Fc, samples eluted from affinity columns were electrophoretically separated on reducing and denaturing 7% NuPAGE Tris-acetate polyacrylamide gels, stained with Coomassie Blue dye, destained and photographed for comparison with known molecular weight markers run on the same gel.

Sequence Analysis

Recombinant polypeptides separated by polyacrylamide gel electrophoreses (PAGE) and were blotted onto PVDF membranes;protein bands visualized by rapid Ponceau S staining and de-staining were excised from the membranes in preparation for Edman degradation (AIBioTech, Richmond VA) to yield N-terminal sequence information. The amino terminal sequences obtained were aligned with known sequences from either the mature N-terminus of vWF or from the N-terminal propeptide sequence that appeared in bands expected to represent incompletely processed vWF products.

In vitro Association of BDD-FVIII and the truncated vWF-Fc proteins

Once cells expressing the D'-D3-Fc, Pro-D'-D3-Fc, D'-A1-Fc, Pro-D'-A1-Fc, D'-A3-Fc, and Pro-D'-A3-Fc proteins were selected and propagated, samples of cell supernatant were clarified and added to an actively-growing BDD-078 cells (a mammalian cell clone expressing B-domain-deleted FVIII). BDD-078 cells were seeded at 12.5 x 10^6 cells/ml with PER.C6 cell supernatants from cells transfected with the plasmid constructs for expression of the truncated vWF-Fc fusion proteins (*i.e.*, D'-D3-Fc, Pro-D'-D3-Fc, D'-A1-Fc, Pro-D'-A1-Fc, D'-A3-Fc, Pro-D'-A3-Fc). For the "BDD Ctrl," conditioned medium was substituted. Cells were grown for 2 days with shaking at 37°C as described. At that point, aliquots were removed, centrifuged and supernatants were tested for both FVIII activity as well as FVIII and vWF antigen. Only FVIII activity is shown in Figure 8. The dash bar indicates the increase above the control BDD-FVIII without added vWF-Fc proteins.

Under normal conditions, the BDD-FVIII expressed from BDD-078 cells appeared to be catabolized or sequestered by the cells (Kalind *et al.*, *J. Biotechnology*, 147:198-204 (2010)). However, addition of either plasma-derived vWF protein, or of supernatants of cells expressing one of the truncated vWF-Fc fusion proteins, resulted in higher recovery of FVIII activity, suggesting a protective effect against unwanted uptake or catabolism of FVIII by growing cells. This result demonstrates that the truncated vWF-Fc fusions can function to increase FVIII yield in culture. After 2 days in 37°C culture with the expressed FVIII, aliquots were taken and evaluated for accumulation of FVIII activity. A substantial increase in recovered FVIII activity was observed (Figure 8).

Multimer Analysis

Plasma-derived vWF (pd-vWF) and the recombinant polypeptides were evaluated for their ability to form high molecular weight complexes by electrophoretic analysis on non-reducing, 1.6 or 2% high-melting temperature (HGT-P) agarose gels (modified from Raines *et al.*, *Thrombosis Res.*, 60:201-212 (1990),. Plasma-derived FVIII (Koate-DVI®) was used as electrophoresis standard for evaluation of vWF-Fc multimers. Proteins were transferred by semi-dry blotting methods to nitrocellulose paper on an iBlot device (Invitrogen Corp., Rockville, MD) and blocked using SuperBlock Solution (Pierce, Rockford, IL). Samples were incubated with a rabbit anti-humanvWF polyclonal antibody (Abcam cat# ab6994) followed by an alkaline phosphatase-conjugated goat anti-rabbit IgG $F(ab')_2$ fragment (cat# A3937; Sigma); detection of bands was by incubation with Western Blue solution (Invitrogen Corp., Carlsbad, CA). A rinse in distilled water was used to quench the reaction; and bands were visualized on a BioRad Molecular Imager ChemiDoc XRS$^+$ Imaging System (Bio-Rad Laboratories Hercules, CA). The results are shown in Figure 9.

Protein Purification and Chromatography on Protein A

Clarified supernatants from cell cultures expressing BDD-FVIII and pro-D'-A3-Fc protein were prepared by triple centrifugation and separation at 2,500 x g for 7 minutes, followed by 2,500 x g for 11 minutes. Supernatants were then filtered through a Sartobran 150 depth filter (0.45 micrometer), followed by a 0.2 micrometer, cellulose acetate filter (#5231307-H4-00) (Sartorius Stedim Stedim Biotech S.A., Aubagne, France). Filters were pre-wetted using a pump

with 100-200 ml of 20 mM Tris, pH 7.0, followed by cell supernatant, and finally flushed with ca.25 ml of 20 mM Tris, pH 7.0. The final filtrate was two-fold-diluted cell supernatant and was the material used for column chromatography. Filtered supernatants were applied to a 5 or 10 milliter Protein A-HiTrap column (part 17-1403-01) (GE Healthcare, Piscataway, NJ) on an AKTA Explorer Chromatography System (GE Healthcare, Piscataway, NJ). System tubing was pre-flushed with 20mM Tris, pH 7.0, and the Protein A column was washed with five column volumes of 20mM Tris, pH 7.0 to ensure a stable baseline before application of sample to the column. Filtered samples were run through the column at 5 milliliter/min, and the eluate was collected as "flow-through". Once all material was loaded, the Protein A-HiTrap column was additionally washed with five column volumes of 20mM Tris, pH 7.0 until a stable baseline was achieved. At this point, the column was washed with four column volumes of 20mM Tris, pH 7.0 containing 0.1 M $CaCl_2$ and eluted material was collected. The Factor VIII bound to the truncated vWF-Fc fusions was then eluted with 20mM Tris, pH 7.0 containing 0.3 M $CaCl_2$ until the fraction eluted (approximately three column volumes). The column was then washed with five additional column volumes of 20mM Tris, pH 7.0. The truncated vWF-Fc proteins remaining bound to protein A were stripped from the column by addition of 250mM Glycine, 150mM NaCl, pH 3.9. Alternative elution methods have been described, *e.g.,* Arakawa *et al.*, *Prot. Expr. Purif.*, 63:158-163 (2009). All collected samples from the Protein A-HiTrap column were saved, tested for Factor VIII activity using the chromogenic and/or clotting assay (described above) and aliquots were prepared for SDS-PAGE electrophoresis. In some cases, for example where FVIII peak activity was detected, the protein was loaded onto PD10 columns (GE Healthcare 45000148) that were pre-washed with 25 ml of desired FVIII storage buffer. Two and one-half milliliters of eluted BDD-FVIII was then applied to each column and the desalted BDD-FVIII was eluted with 3.5 ml FVIII storage buffer. Proteins were then placed at -80°C for long-term storage; and, in some cases, serum albumin was added to 10 mg/ml.

Purification of FVIII from pro-D'-A3-Fc/FVIII Binding Complex

To determine whether the truncated vWF-Fc polypeptides were capable of binding FVIII, cells co-expressing BDD-FVIII and pro-D'-A3-Fc were grown for 5 days in PER-MAb media with 125 micrograms/ml each of neomycin and zeocin. Supernatants were prepared and chromatographed substantially as described above. At various points during elution, samples

were saved and electrophoresed (Figure 10). In Figure 10A, chromatographic trace of peaks eluted with different buffer conditions after application of 10 ml of BDD-FVIII/proD'A3-Fc PER.C6 cell supernatant onto a 5 ml HiTrap Protein A column is shown (step 5 is a wash step, step 6 is the eluate after 0.1 M $CaCl_2$, step 7 is the FVIII eluate after 0.3M $CaCl_2$ wash, step 8 is the eluate after 1 M $CaCl_2$ wash, step 9 is a low salt wash, and step 10 is the eluate after stripping the column with citrate, pH 5.5). Figure 10B shows polyacrylamide gel electrophoresis of the elution samples. Lanes 1-9 represent, respectively, (1) starting material, (2) flow-through , (3) 0.1M $CaCl_2$ eluate, (4) 0.3M $CaCl_2$ eluate, (5) pH 5.5 citrate eluate, (6) a concentrated BDD-FVIII preparation, (7) molecular weight markers (sizes on right side), (8) cell supernatant from pro-D'-A3-Fc expressing PER.C6 cells, (9) commercial BDD-FVIII (Xyntha®), asterisks showing three bands of 170, 90, and 80 kd, corresponding to full-length BDD-FVIII, heavy chain and light chain(s), respectively.

Dots adjacent to bands in lanes 5 and 8 show migration of expected sizes for propeptide-containing proD'-A3-Fc and mature proD'-A3-Fc proteins (higher and lower molecular weights, respectively).

A substantially pure fraction of BDD-FVIII bound to pro-D'A3-Fc was captured on protein A matrix. The complex was stable to washing conditions before being separated and eluted from the vWF-Fc matrix only at the 0.3 M $CaCl_2$ elution step (Figure 10A, step 7; and Figure 10B, lane 4). The pro-D'-A3-Fc protein itself is only visualized by stripping the matrix under harsher washing conditions that included an acidic pH of 3.9 (Figure 10A, step 10; and Figure 10B, lane 5). The proteins eluted by the pH 3.9 wash co-migrate with a set of proteins that previously were prepared and identified as authentic truncated pro-D'-A3-Fc molecules (Figure 10B, lane 8).

Purification of FVIII from D'-D3-Fc Binding Complex

A 10 ml solution of PER.C6 cell supernatant containing expressed pro-D'-D3-Fc protein (Fig. 11, lane 2) was added to 250 units of commercial BDD-FVIII (Xyntha®, lane 3) and incubated at 37°C for 4 hours (lanes 4 and 5). The mixture was diluted two-fold with 20 mM Tris-HCl, pH 7.0 and applied to a 1 ml Protein A-HiTrap column, essentially as described for purification of FVIII from pro-D'-A3-Fc complexes above and shown in Figure 10. Unbound proteins were washed off the column with 20 mM Tris-HCl, pH 7.0 (lane 6). There is clear retention of FVIII complexed with pro-D'-D3 protein, as seen by lack of FVIII and/or vWF-Fc

polypeptides in Fig 11, lane 6 (as compared to lane 7), followed by dramatic elution of FVIII (lane 7) with 0.3 M $CaCl_2$; this compares well with the FVIII eluted from FVIII/pro-D'-A3-Fc complexes (Fig. 10B, lane 4). Retained polypeptides representing truncated vWF-Fc are eluted with a low pH buffer, 0.1 M glycine, 0.15 M NaCl, pH 3.9 (Fig. 11, lane 8). . The three asterisks in lane 3 align with proteins bands of approximately 170, 90, and 80 kd, corresponding to full-length BDD-FVIII, heavy chain and light chain(s), respectively. The results from pro-D'D3-Fc/FVIII purification demonstrate that addition of pro-D'D3-Fc with FVIII results in the capture, retention and subsequent specific elution of FVIII, with results virtually identical to those seen in Figure 10

Pharmacokinetics

The purified recombinant vWF-Fc fusion proteins, bound to FVIII or as native protein, is injected intravenously into the tail vein of mice at ca. 5 micrograms/mouse in phosphate-buffered saline with a stabilizing agent, like albumin. As control articles, FVIII with/without vWF is similarly injected and evaluated for clearance in animals. Five to eight animals (either wild-type or having a bleeding disease due to genetic deficiency of either FVIII or vWF, or both) are used for examining blood loss. At different times points (*e.g.*, 0 minutes, 3 min, 15 minutes, 30 minutes, 1 hr, 2 hr, 4 hr, 8 hr, 16 hr, 24 hr) post-injection, animals are sacrificed, blood is withdrawn through the inferior *vena cava*, and plasma is prepared and frozen. Plasma samples are then evaluated for antigen and/or functional activity by ELISA and/or chromogenic or clotting assays, respectively, depending on the genetic background of the animal. Plasma clearance is determined by pharmacokinetic analysis of antigen and/or activity vs. time (*e.g.* as described by Mordenti *et al.*, Toxicol. Appl. Pharmacol., 137:75-78 (1996) and Lenting *et al.*, J. Biol. Chem., 279:12102-12109 (2004), each of which is incorporated herein for its teaching of method for pharmacokinetic analysis), and by comparison with the control article.

We claim:

1. A polypeptide comprising a first amino acid sequence present in a von willebrand factor (vWF) polypeptide and a second amino acid sequence heterologous to the first, wherein the polypeptide is capable of binding a Factor VIII (FVIII), with the proviso that the polypeptide lacks von willebrand factor (vWF) domains D4, B1, B2, B3, C1, C2 and CK, wherein the second amino acid sequence is C-terminus to the first amino acid sequence, , wherein the first amino acid sequence is as set forth in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14 or SEQ ID NO:15, and wherein the second amino acid sequence corresponds to an immunoglobulin Fc.

2. The polypeptide of claim 1, wherein the polypeptide comprises the sequence set forth in SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21 or SEQ ID NO:22.

3. The polypeptide of claim 1, wherein the polypeptide is capable of forming a dimer.

4. The polypeptide of claim 1, wherein the second amino acid sequence has the sequence set forth in SEQ ID NO:16.

5. A composition comprising the polypeptide of claim 1.

6. A protein complex comprising the polypeptide of claim 1, and a Factor VIII (FVIII) polypeptide.

7. A composition comprising the protein complex of claim 6.

8. A nucleotide sequence encoding the polypeptide of claim 1.

9. The nucleotide sequence of claim 8, wherein the nucleotide sequence is set forth in SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27 or SEQ ID NO:28.

10. An expression vector comprising the nucleotide sequence of claim 8.

11. A cell expressing the polypeptide of claim 1.

12. A cell expressing the protein complex of claim 6.

13. An *in vitro* method for preparing the protein complex of claim 6, the method comprising contacting the polypeptide with the Factor VIII (FVIII).

14. The method of claim 13, wherein the contacting comprises recombinantly expressing the polypeptide in a cell that expresses the Factor VIII (FVIII).

15. An *in vitro* method for binding a Factor VIII (FVIII), the method comprising: contacting a Factor VIII (FVIII) polypeptide with a polypeptide of claim 1 to form a protein complex comprising the polypeptide of claim 1 and the Factor VIII (FVIII).

16. The method of claim 15 further comprising selectively adhering the complex to a resin comprising a binding partner having affinity for a domain defined by the second amino acid sequence.

17. The method of claim 16, wherein the binding partner is a protein A or a protein G.

18. Use of a composition comprising the protein complex of claim 6, in the manufacture of a medicament for enhancing a plasma pharmacokinetic property of a Factor VIII (FVIII).

19. The use of claim 18, wherein the property is extended plasma half-life.

20. A composition comprising the protein complex of claim 6, and a pharmaceutically acceptable carrier.

21. Use of the composition of claim 20 in the manufacture of a medicament for treating hemophilia.