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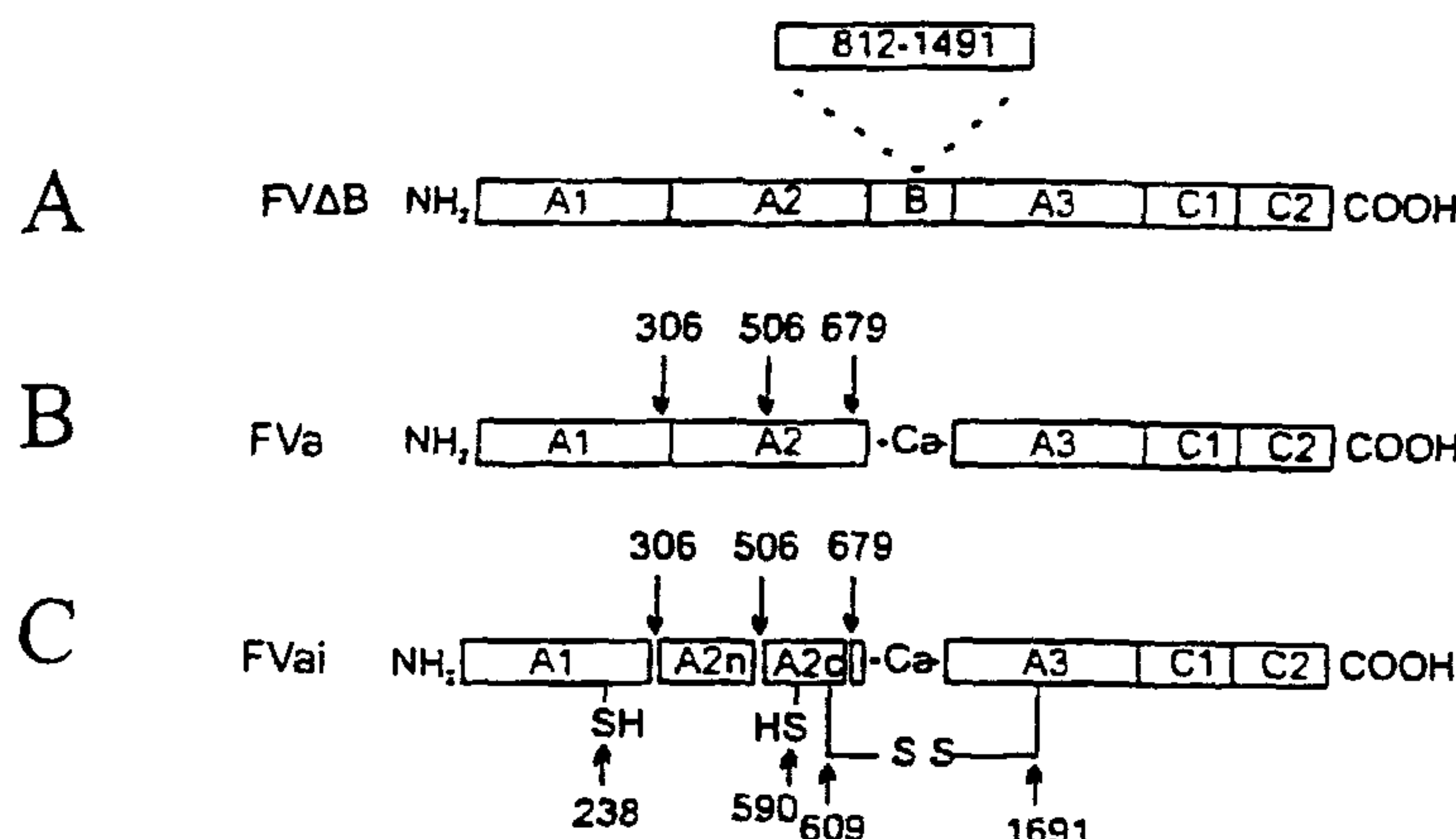
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(54) Title: STABILIZED PROTEINS WITH ENGINEERED DISULFIDE BONDS



(57) Abrégé/Abstract:

The present invention relates to methods of introducing one or more cysteine residues into a polypeptide which permit the stabilization of the polypeptide by formation of at least one bond, preferably a disulfide bond, between different domains of the polypeptide. The invention also relates to polypeptides containing such introduced cysteine residue(s), nucleic acids encoding such polypeptides and pharmaceutical compositions comprising such polypeptides or nucleic acids. The invention also relates to vectors, viral particles and host cells containing such nucleic acids, and methods of using them to produce the polypeptides of the invention. Exemplified polypeptides include plasma proteins, including hepatocyte growth factor activator and plasma hyaluronin binding protein, as well as blood coagulation factors, such as Factor VIII, Factor V, Factor XII and prothrombin.



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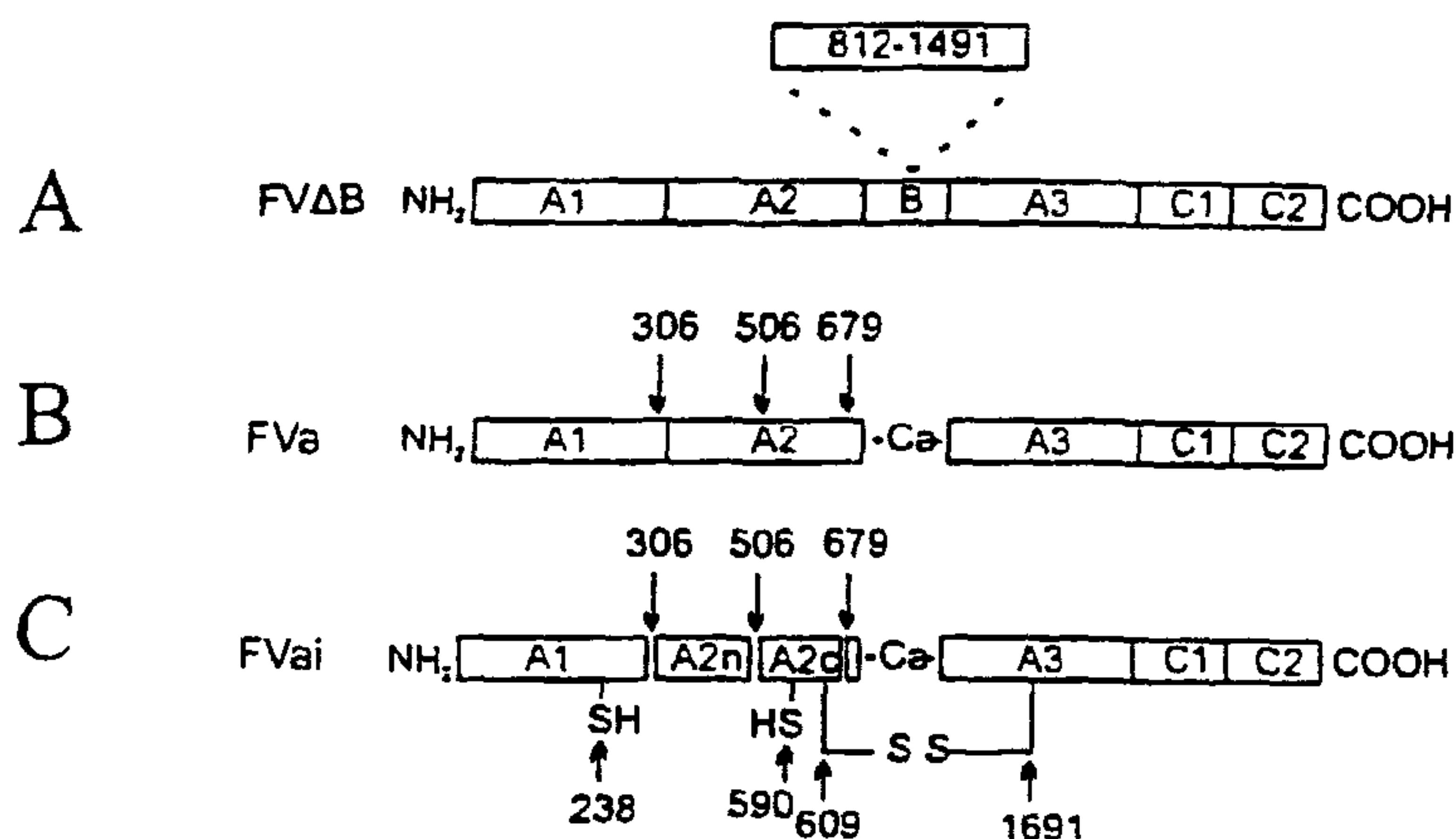
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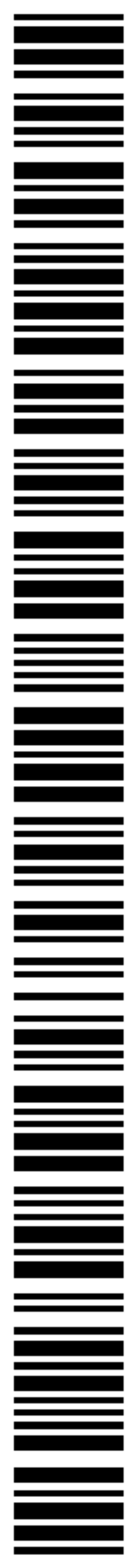
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(54) Title: STABILIZED PROTEINS WITH ENGINEERED DISULFIDE BONDS



(57) Abstract: The present invention relates to methods of introducing one or more cysteine residues into a polypeptide which permit the stabilization of the polypeptide by formation of at least one bond, preferably a disulfide bond, between different domains of the polypeptide. The invention also relates to polypeptides containing such introduced cysteine residue(s), nucleic acids encoding such polypeptides and pharmaceutical compositions comprising such polypeptides or nucleic acids. The invention also relates to vectors, viral particles and host cells containing such nucleic acids, and methods of using them to produce the polypeptides of the invention. Exemplified polypeptides include plasma proteins, including hepatocyte

growth factor activator and plasma hyaluronin binding protein, as well as blood coagulation factors, such as Factor VIII, Factor V, Factor XII and prothrombin.



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TITLE

STABILIZED PROTEINS WITH ENGINEERED DISULFIDE BONDS

This invention was made with the assistance of funds provided by the Government of the United States. The government may own certain rights in the present invention, pursuant to grants from the National Institutes of Health, grant numbers R01HL21544, R37HL52246, T32HL07695 and P01GM48495.

FIELD OF THE INVENTION

The present invention relates to methods of introducing one or more cysteine residues into a polypeptide which permit the stabilization of the polypeptide by formation of at least one bond, preferably a disulfide bond, between different domains of the polypeptide. The invention also relates to polypeptides containing such introduced cysteine residue(s), nucleic acids encoding such polypeptides and pharmaceutical compositions comprising such polypeptides or nucleic acids. The invention also relates to vectors, viral particles and host cells containing such nucleic acids, and methods of using them to produce the polypeptides of the invention.

BACKGROUND OF THE INVENTION

Many polypeptides are known which are the expression product of a single gene. A number of these polypeptides are originally synthesized as a single polypeptide chain, but contain multiple, independently folded domains, which are subject to limited proteolysis (or proteolytic cleavage(s)) in vivo that may result in separation of domains due to dissociation of the cleavage products. Proteolysis resulting in the separation of domains has been shown to alter the stability and/or enzymatic or functional activities of a variety of these proteins. Examples of these proteins include plasma proteins, such as those involved in blood coagulation.

As known in the art, blood clotting begins when platelets adhere to the wall of an injured blood vessel at a lesion site. Subsequently, in a cascade of enzymatically regulated reactions, soluble fibrinogen molecules are converted by the enzyme thrombin to insoluble strands of fibrin that hold the platelets together in a thrombus. At each step in the cascade, a protease precursor is converted to a protease that cleaves the next protein precursor in the

series. Cofactors are required at most of the steps. In its active form, the protein factor VIII is a cofactor that is required for the activation of factor X by the protease, activated factor IX.

Factor VIII can be activated to factor VIIIa (where "a" indicates "activated") proteolytically by thrombin or factor Xa. In combination with calcium and phospholipid, factor VIIIa makes factor IXa a more efficient activator of factor X by a mechanism which is not fully understood.

People deficient in factor VIII or having antibodies against factor VIII who are not treated with factor VIII suffer uncontrolled internal bleeding that may cause a range of serious symptoms, from inflammatory reactions in joints to early death. Severe hemophiliacs, who number about 10,000 in the United States, can be treated with infusion of factor VIII, which will restore the blood's normal clotting ability if administered with sufficient frequency and concentration.

Several preparations of human plasma-derived or recombinant factor VIII of varying degrees of purity are available commercially for the treatment of hemophilia A. These include a partially-purified factor VIII derived from the pooled blood of many donors that is heat- and detergent-treated for viruses but contains a significant level of antigenic proteins; a monoclonal antibody-purified factor VIII that has lower levels of antigenic impurities and viral contamination; and recombinant human factor VIII.

Hemophiliacs require daily replacement of factor VIII to prevent the deforming hemophilic arthropathy that occurs after many years of recurrent hemorrhages into the joints. However, supplies of factor VIII concentrates have never been plentiful enough for treating hemophiliacs adequately because of problems in commercial production and therapeutic use. For example, the commonly used plasma-derived factor VIII is difficult to isolate and purify, is immunogenic, and requires treatment to remove the risk of infectivity from AIDS and hepatitis viruses. Porcine factor VIII may also present an alternative, however a limitation of porcine factor VIII is the development of inhibitory antibodies to it after one or more infusions.

Activated factor VIII (FVIIIa) is thermodynamically unstable under physiological conditions due to the tendency of the A2 domain to dissociate from the rest of the complex. In other words, activated FVIII spontaneously becomes inactive. If this dissociation could be prevented in pharmacological preparations of FVIII or FVIIIa, administration that is less frequent and/or of lower concentration, could be realized. This

could result in a number of benefits such as cost savings, decreased use of medical personnel, and improved lifestyle for hemophiliacs.

Another plasma protein besides factor VIII is prothrombin. As part of the coagulation cascade, prothrombin is converted to thrombin by the action of the prothrombinase complex (FXa, FVa, and Ca^{2+}). In human prothrombin, this conversion involves cleavages at Arg271 and Arg284, between the F2 domain and the thrombin A chain, and at Arg320, between the A and B chains (human numbering system). *In vivo*, prothrombinase first cleaves prothrombin at Arg320, producing meizothrombin. Free meizothrombin is an unstable intermediate, and autolysis at the Arg155-Ser156 bond rapidly removes the F1 domain to generate meizothrombin (des F1), which slowly converts to thrombin via the cleavages at Arg271 and Arg284. In the presence of thrombomodulin and phosphatidylserine/phosphatidylcholine phospholipid vesicles (PCPS), meizothrombin and meizothrombin (des F1) are better activators of protein C than thrombin (41, 42).

An additional plasma protein is factor V. Human coagulation factor V (FV) is a 330,000 MW protein, which is composed of six domains of three types in the order A1-A2-B-A3-C1-C2 (4). FV is cleaved by thrombin to remove most of the B domain and produce activated FV (FVa). Human FVa is composed of a heavy chain (A1-A2, residues 1-709) and a light chain (A3-C1-C2, residues 1546-2196), which form a non-covalent complex (5). FVa is the nonenzymatic cofactor for factor Xa (FXa) in the prothrombinase complex, which converts prothrombin to thrombin, in the presence of negatively charged phospholipids (6). Inactivation of FVa is a complex process involving APC (activated Protein C) cleavages of FVa at Arg506, Arg306 and Arg679. Cleavage at Arg506 is faster than cleavage at Arg306, and it only partially inactivates FVa while cleavage at Arg306 completely inactivates FVa and causes dissociation of the A2 domain fragments (7-10). Fully inactive FVa loses the ability to bind to FXa (11).

Still another plasma protein is factor XII. Human FXII is a single-chain protein with a MW of 76,000 and 596 amino acids. It contains, in order from N-terminus to C-terminus fibronectin type II domain, EGF domain, fibronectin type I domain, EGF domain, Kringle domain, trypsin-like serine protease domain. At least two forms of activated factor XII (FXIIa) exist. α FXIIa is formed by cleavage of the bond following Arg353, generating a two chain molecule comprised of a heavy chain (353 residues) and a light chain (243 residues) held together by a disulfide bond. Further cleavage results in FXIIa (FXIIa fragment). This is the result of cleavage at Arg334 and Arg343, resulting in two polypeptide

chains (9 and 243 residues) held together by a disulfide bond (43, 44). The bulk of the N-terminal heavy chain fragment is no longer associated. Negative surface/membrane binding is mediated through this heavy chain so the resulting FXIIa fragment no longer binds to surfaces but it is still catalytically active.

The protein HGFA (hepatocyte growth factor activator) has the same domain structure as FXII (45) and is also activated by proteolytic cleavage, in this case, only one cleavage by thrombin at Arg407 (46), homologous to Arg353 in FXII. But further cleavage by kallikrein at Arg372 also results in release of the N-terminal heavy chain, which, as in FXII, is involved in surface binding (47). As known in the art, HGFA activates hepatocyte growth factor (HGF) within injured tissues where HGF plays roles in tissue repair via a mitogenic activity towards a variety of cell types.

- Another FXII-like polypeptide is known by two names: PHBP (plasma hyaluronin binding protein) (48) and FVII activating protease (49). PHBP is a serine protease and is homologous to HGFA though the domain structure is not exactly the same (49, 50). This protein activates FVII, uPA, and tPA in experimental systems, but the physiological role has not been established (49, 50).

SUMMARY OF THE INVENTION

According to embodiments of the present invention, one may engineer into a polypeptide one or more cysteine residues to permit formation of a bond, such as a disulfide bond, between two or more of the polypeptide's domains. Placement of such disulfide bond(s) allows one to achieve results such as polypeptide stabilization. Such stabilization can result in the prolonged retention of desired activities of the undissociated polypeptide or the avoidance of undesired activities of the disassociated polypeptide.

Preferred polypeptides useful in the invention are those which are synthesized in nature as a single polypeptide chain, generally as the expression product of a single gene, and which contain multiple, independently folded domains which are subject to limited proteolysis that may result in separation of domains due to dissociation. Examples of such polypeptides include plasma proteins, including hepatocyte growth factor activator and plasma hyaluronin binding protein, as well as blood coagulation factors, such as Factor VIII, Factor V, Factor XII and prothrombin.

Mutant polypeptides of the invention (i.e., those polypeptides into which one or more cysteine(s) have been introduced) include not only those in which the domains which

are linked are synthesized from a single nucleic acid sequence (e.g., from a single gene, cDNA, or synthetic or semi-synthetic coding sequence), but also those in which the domains which are linked are synthesized from distinct (or separate) nucleic acid sequences (e.g., from sequences encoding polypeptides comprising each of the linked domains, which sequences may or may not be present on a contiguous nucleic acid molecule). In the latter case, the domains may be joined together after synthesis, either *in vivo* or *in vitro*.

Preferred mutant polypeptides of the invention are those which have increased stability and/or retain desirable enzymatic or functional activities for a longer period of time as compared to the corresponding unmutated polypeptide.

One aspect of the invention relates to a method of stabilizing a polypeptide which is the product of a single gene in nature by introducing one or more cysteines comprising the steps of: (a) obtaining or creating a three-dimensional structure of the polypeptide; (b) predicting one or more sites for the introduction of one or more cysteines based on the three dimensional structure; and (c) creating one or more mutants of said polypeptide by introducing one or more cysteines at one or more of the predicted sites; wherein the introduction of said one or more cysteines permits the formation of at least one intramolecular, interdomain disulfide bridge which increases the stability of the mutant polypeptide as compared to that of the polypeptide which does not contain said introduced one or more cysteines.

Another aspect of the invention relates to a polypeptide which is the product of a single gene in nature which has been mutated by introducing at least one cysteine, wherein the introduction of said cysteine permits the formation of at least one intramolecular, interdomain disulfide bridge with another cysteine, which increases the stability of the mutant polypeptide as compared to that of the polypeptide which does not contain said introduced cysteine.

Another aspect of the invention relates to compositions comprising the polypeptides of the invention, including pharmaceutical compositions comprising the polypeptides of the invention and a pharmaceutically acceptable carrier.

The invention also relates to nucleic acids coding for the polypeptides of the invention, including vectors containing such nucleic acids. The invention also relates to viral particles containing such nucleic acids and/or vectors. The invention also relates to host cells containing such nucleic acids, vectors, and viral particles. The invention also relates to

compositions (including pharmaceutical compositions) which contain the nucleic acids, vectors, viral particles and/or host cells of the invention.

The invention also relates to methods of treating individuals with the polypeptides, nucleic acids, vectors, viral particles or host cells of the invention and/or pharmaceutical compositions thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a schematic of recombinant B domain-deleted FV molecules. Fig. 1A is a schematic of the primary sequence of FV Δ B (B-domain deleted human Factor V) with the locations of the different domains indicated. Fig. 1B is a schematic showing activated FV Δ B (FVa), a heterodimer of the N-terminal heavy chain and the C-terminal light chain associated in the presence of Ca²⁺ ions. Arrows indicate sites of cleavage in FVa by APC. Fig. 1C is a schematic showing the cleavage fragments produced upon inactivation of FVa (FVai) by APC, and further shows the sites of cysteine mutations that did (His609-Glu1691) and did not (Leu238-Gln590) result in disulfide bond formation.

Fig. 2. Immunoblots of various FVa and FVai mutants. (A) Immunoblot developed with an anti-FV light chain monoclonal antibody. Samples in lanes 1 through 6 were not reduced and those in lanes 7 through 12 were reduced. Lanes 1 and 7, 2183A-FVa; lanes 2 and 8, 2183A-FVai; lanes 3 and 9, A2-SS-A3-FVa; lanes 4 and 10, A2-SS-A3-FVai; lanes 5 and 11, Q506-A2-SS-A3-FVa; lanes 6 and 12, Q506-A2-SS-A3-FVai. (B) Immunoblots developed with anti-FV heavy chain polyclonal antibodies. Lane 1, non-reduced A2-SS-A3-FVa; lane 2, non-reduced A2-SS-A3-FVai; lane 3, reduced A2-SS-A3-FVa; lane 4, reduced A2-SS-A3-FVai; lane 5, non-reduced Q506-A2-SS-A3-FVa; lane 6, non-reduced Q506-A2-SS-A3-FVai; lane 7, reduced Q506-A2-SS-A3-FVa; lane 8, reduced Q506-A2-SS-A3-FVai. Band positions for cross-linked and non cross-linked fragments are indicated on the right side of each blot. LC = light chain, HC = heavy chain, A1 = A1 domain, A2 = A2 domain, A2c = C-terminal fragment of the A2 domain (residues 507-679). Molecular weight marker positions (kDa, Novex SeeBlue standards) are indicated on the left side.

Fig. 3 is a schematic illustrating the prevention of dissociation of the A2 domain from heterotrimeric Factor VIIIa by introduction of a disulfide bond between the A2 and A3 domains, or the A2 and A1 domains, of FVIIIa.

Fig. 4 is a schematic showing the expected action of APC upon mutant FVIIIa containing both a disulfide bridge between cysteine residues introduced at positions corresponding to Met 662 - Asp 1828 in one mutant or Tyr 664 - Thr 1826 in another mutant, and showing the APC cleavage sites at residues Arg 336 and Arg 562.

Fig. 5. Stability of Double Cysteine mutants of Factor VIIIa. Recombinant wildtype and double cysteine mutants of FVIIIa were assayed over time for activity in an APTT assay. FVIIIa species as indicated: wildtype (+), C662-C1828 (Δ), C664-C1826 ($\circ$). At the start time about 500 mU/mL FVIII was treated with 5.4 nM thrombin and then after one minute hirudin was added to 1 U/mL to inactivate the thrombin. Then samples were removed at indicated times and assayed for remaining FVIIIa activity in the APTT assay.

Fig. 6 is a schematic illustrating the introduction of a disulfide bond into human prothrombin to stabilize its meizothrombin or meizothrombin (des F1) form, preventing the conversion of meizothrombin or meizothrombin (des F1) to alpha-thrombin (α -IIa). Legend: GLA, Gla domain; Kr.1, kringle 1 domain; Kr.2, kringle 2 domain; Meizo-IIa, meizothrombin. Prothrombin and Meizo-IIa are shown with an introduced disulfide bond between the Kringle 2 domain and the protease domain formed from the introduction of a cysteine at a residue N-terminal to its cleavage site at residue 271 in the Kringle 2 domain and the introduction of a cysteine at a residue C-terminal to the cleavage site at residue 320 in the protease domain. The disulfide bond between cysteine residues 293 and 439 is present in the naturally occurring protein.

Fig. 7 is a description of the accession numbers and related references used as a source for the amino acid sequences, with notes concerning the numbering system for Factor VIII, Factor V, Prothrombin, Factor XII, HGFA and PHBP mutants described in the examples herein.

Fig. 8. Webpages from SwissProt Accession # P00451 containing amino acid sequence of human Factor VIII and related information.

Fig. 9. Webpages from SwissProt Accession # P12259 containing amino acid sequence of human Factor V and related information.

Fig. 10. Webpages from SwissProt Accession # P00734 containing amino acid sequence of human Prothrombin and related information.

Fig. 11. Webpages from SwissProt Accession # P00748 containing amino acid sequence of human Factor XII and related information.

Fig. 12. Webpages from SwissProt Accession # Q04756 containing amino acid sequence of human HGFA and related information.

Fig. 13. Webpages from PIR Accession # JC4795 containing amino acid sequence of human PHBP and related information.

DETAILED DESCRIPTION OF THE INVENTION

It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only, and are not restrictive of the invention, as claimed. The accompanying drawings, which are incorporated in and constitute a part of the specification, illustrate an embodiment of the invention and, together with the description, serve to explain the principles of the invention.

General Procedure

According to embodiments of the present invention, one may engineer into a polypeptide, such as the polypeptide product of a single gene, one or more cysteine residues which permit the formation of a disulfide bond between two or more of the polypeptide's domains. Placement of such cysteine(s), with their resultant, disulfide bonds allows one to achieve results such as polypeptide stabilization. It is noted that, in some embodiments, the present invention may also be used to place a disulfide bond within a single domain of a polypeptide, between two different polypeptides, and the like.

As a first step, a structure of the polypeptide of interest is obtained or created. This may be an x-ray crystal structure, an NMR-derived structure, a three-dimensional structure based on homology modeling, neutron diffraction or the like.

Next, an algorithm which predicts sites for the introduction of disulfide bridges by placement of cysteines may be applied to a structure of the polypeptide of interest. This may be done, for example, by using the computer program MODIP which employs the algorithm of Sowdhamini (19). MODIP predicts sites for the introduction of disulfide bridges, and provides grades (A, B, C) for each prediction. Grade A sites are those predicted to be most optimal for the establishment of disulfide bridges, while grade B and grade C sites are progressively less ideal. Said differently, grade A disulfide bridges satisfy defined stereochemical criteria while grade C disulfides satisfy fewer of the stereochemical criteria. It is specifically noted that other algorithms and/or computer programs, such as the algorithm of Pabo (18) or Hazes (56) may be used. In other embodiments, predictions for the

introduction cysteines in order to establish disulfide bridges may be made by other methods such as by visual inspection.

Of the sites predicted, one may choose a number of the most ideal sites for further investigation.

Visual inspection of the chosen sites may be performed using computational graphics analysis. Based on this visual inspection, certain sites may be eliminated from further consideration. For each of the chosen sites remaining in consideration after visual inspection, a modified structural model including a disulfide bond at the chosen site may be created. This may be done using computer programs, such as the Xfit computer program, for example, with refinement being provided by another computer program, for example, the X-PLOR computer program using the Charm22 all atoms force field.

After refinement, the modeled disulfide bonds may be analyzed for optimal disulfide geometry. Those sites with the best geometry for formation of disulfide bonds, and perhaps the lowest Van Der Waals gas phase energies, may be chosen for attempts to introduce one or more cysteine residues which permit the formation of one or more disulfide bonds. Cysteine residues may be introduced into a polypeptide using techniques well known in the art such as, for example, recombinant techniques such as site directed mutagenesis of a nucleic acid encoding the polypeptide of interest. Nucleic acids encoding polypeptides of the invention may also be made by synthetic or semi-synthetic methods. For example, the nucleic acid encoding the polypeptide of the invention can be synthesized directly using overlapping synthetic deoxynucleotides (see, e.g., Edge et al., Nature 292:756 (1981); Nambair et al., Science 223:1299 (1984); Jay et al., J. Biol. Chem. 259:6311 (1984); or by using a combination of polymerase chain reaction generated DNAs or cDNAs and synthesized oligonucleotides. The nucleic acids of the invention can be present in, or inserted into an expression vector containing an appropriate promoter region operably linked to the sequence encoding the polypeptide of the invention and an appropriate terminator signal. Afterwards, vector purification, and transfection procedures known in the art may be performed. Next, stable clones may be selected and collected using methods known in the art. Produced polypeptides may then be quantified by activity and by immunoblots so as to confirm the proper placement of the disulfide bond(s) in the polypeptide of interest and the yields thereof.

Nucleic acids encoding the polypeptides of the invention can be expressed in the native host cell or organism or in a different cell or organism. The nucleic acids can be

introduced into a vector such as a plasmid, cosmid, phage, virus or mini-chromosome and inserted into a host cell or organism by methods well known in the art. In general, the nucleic acids or vectors containing these nucleic acids can be utilized in any cell, either eukaryotic or prokaryotic, including mammalian cells (e.g., human (e.g., K293, HeLa), monkey (e.g., COS), rabbit (e.g., rabbit reticulocytes), rat, hamster (e.g., CHO and baby hamster kidney cells) or mouse cells (e.g., L cells), plant cells, yeast cells, insect cells or bacterial cells (e.g., *E. coli*). The vectors which can be utilized to clone and/or express these nucleic acids encoding the polypeptide are the vectors which are capable of replicating and/or expressing the nucleic acids in the host cell in which the nucleic acids are desired to be replicated and/or expressed. See, e.g., F. Ausubel et al., Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley-Interscience (1992) and Sambrook et al. (1989) for examples of appropriate vectors for various types of host cells. The native promoters for such genes can be replaced with strong promoters compatible with the host into which the nucleic acid encoding the polypeptide of the invention is inserted. These promoters may be inducible. The host cells containing these nucleic acids can be used to express large amounts of the polypeptides of the invention useful in enzyme preparations, pharmaceuticals, diagnostic reagents, and therapeutics. The polypeptides of the invention may also be made in transgenic plants or animals using methods known in the art.

If the genes which naturally encode the polypeptides of the invention contain inhibitory/instability regions (see, e.g., WO 93/20212) less-preferred codons may be altered to more-preferred codons. If desired, however, (e.g., to make an AT-rich region more GC-rich), more-preferred codons can be altered to less-preferred codons. Optionally, only the most rarely used codons (identified from published codon usage tables, such as in T. Maruyama et al., Nucl. Acids Res. 14(Supp):r151-197 (1986)) may be replaced with preferred codons, or alternatively, most or all of the rare codons can be replaced with preferred codons. Generally, the choice of preferred codons to use will depend on the codon usage of the host cell in which the altered gene is to be expressed. Note, however, that the substitution of more-preferred codons with less-preferred codons is also functional.

As noted above, coding sequences are chosen on the basis of the genetic code and, preferably on the preferred codon usage in the host cell or organism in which the nucleic acid encoding a polypeptide of this invention is to be expressed. In a number of cases the preferred codon usage of a particular host or expression system can be ascertained from available references (see, e.g., T. Maruyama et al., Nucl. Acids Res. 14(Supp):r151-197

(1986), in which the number of times the codon appears in genes per 1000 codons is listed in parentheses next to the codon), or can be ascertained by other methods (see, e.g., U.S. Patent No. 5,082,767 entitled "Codon Pair Utilization", issued to G.W. Hatfield et al. on January 21, 1992). Preferably, sequences will be chosen to optimize transcription and translation as well as mRNA stability so as to ultimately increase the amount of polypeptide produced.

Selection of codons is thus, for example, guided by the preferred use of codons by the host cell and/or the need to provide for desired restriction endonuclease sites and could also be guided by a desire to avoid potential secondary structure constraints in the encoded mRNA transcript. Potential secondary structure constraints can be identified by the use of computer programs such as the one described in M. Zucker et al., Nucl. Acids Res. 9:133 (1981). More than one coding sequence may be chosen in situations where the codon preference is unknown or ambiguous for optimum codon usage in the chosen host cell or organism. However, any correct set of codons would encode the desired protein, even if translated with less than optimum efficiency. Example III of Seed et al., U.S. Patent No. 6,114,148, describes a synthetic Factor VIII gene (encoding B-domain deleted Factor VIII), with altered codon usage which increases the expression of the encoded Factor VIII polypeptide.

It is also anticipated that inhibitory/instability sequences can be mutated such that the encoded amino acids are changed to contain one or more conservative or non-conservative amino acids yet still provide for a functionally equivalent protein. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a neutral substitution in the amino acid sequence. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

Nucleic acids for genes altered by the methods of the invention or constructs containing said nucleic acids may also be used for in-vivo or in-vitro gene replacement. For example, nucleic acid which produces a polypeptide without the introduced cysteine residue(s) can be replaced in situ with a nucleic acid that has been modified by the method of the invention in situ to ultimately produce a polypeptide with increased stability as compared

to the originally encoded polypeptide. Such gene replacement might be useful, for example, in the development of a genetic therapy.

Vectors include retroviral vectors and also include direct injection of DNA into muscle cells or other receptive cells, resulting in the efficient expression of the polypeptide of the invention, using the technology described, for example, in Wolff et al., Science 247:1465-1468 (1990), Wolff et al., Human Molecular Genetics 1(6):363-369 (1992) and Ulmer et al., Science 259:1745-1749 (1993). See also, for example, WO 96/36366 and WO 98/34640.

The polypeptides, nucleic acids, vectors, vector particles and/or host cells of the invention can be isolated and purified by methods known in the art and can be used in pharmaceutical compositions and/or therapies as described further below.

Pharmaceutical Compositions

The pharmaceutical compositions of this invention contain a pharmaceutically and/or therapeutically effective amount of at least one polypeptide, or nucleic acid encoding a polypeptide, of this invention. In one embodiment of the invention, the effective amount of polypeptide per unit dose is an amount sufficient to prevent, treat or protect against the effects of a deficiency, or anticipated deficiency, in the corresponding natural polypeptide. The effective amount of polypeptide per unit dose depends, among other things, on the species of mammal treated, the body weight of the mammal and the chosen inoculation regimen, as is well known in the art.

Preferably, the route of inoculation of the peptide will be subcutaneous or intravenous. The dose is administered at least once.

The term "unit dose" refers to physically discrete units suitable as unitary dosages for mammals, each unit containing a predetermined quantity of active material (e.g., polypeptide, or nucleic acid) calculated to produce the desired effect in association with any accompanying diluent.

The polypeptides or nucleic acids of the invention are generally administered with a physiologically acceptable carrier or vehicle therefor. A physiologically acceptable carrier is one that does not cause an adverse physical reaction upon administration and one in which the polypeptides or nucleic acids are sufficiently soluble and retain their activity to deliver a therapeutically effective amount of the compound. The therapeutically effective amount and method of administration of a polypeptide or nucleic acid of the invention may

vary based on the individual patient, the indication being treated and other criteria evident to one of ordinary skill in the art. A therapeutically effective amount of a polypeptide or nucleic acid of the invention is one sufficient to attenuate the dysfunction without causing significant adverse side effects. The route(s) of administration useful in a particular application are apparent to one of ordinary skill in the art.

Routes of administration of the polypeptides and nucleic acids of the invention include, but are not limited to, parenteral, and direct injection into an affected site. Parenteral routes of administration include but are not limited to intravenous, intramuscular, intraperitoneal and subcutaneous. The route of administration of the polypeptides of the invention is typically parenteral.

The present invention includes compositions of the polypeptides and nucleic acids described above, suitable for parenteral administration including, but not limited to, pharmaceutically acceptable sterile isotonic solutions. Such solutions include, but are not limited to, saline and phosphate buffered saline for nasal, intravenous, intramuscular, intraperitoneal, subcutaneous or direct injection into a joint or other area.

A system for sustained delivery of the polypeptide or nucleic acid of the invention may also be used. For example, a delivery system based on containing a polypeptide in a polymer matrix of biodegradable microspheres may be used (57). One such polymer matrix includes the polymer poly(lactide-co-glycolide) (PLG). PLG is biocompatible and can be given intravenously or orally. Following injection of the microspheres into the body, the encapsulated polypeptide is released by a complex process involving hydration of the particles and drug dissolution. The duration of the release is mainly governed by the type of PLG polymer used and the release of modifying excipients (44).

The polypeptides and nucleic acids of the present invention are intended to be provided to the recipient subject in an amount sufficient to prevent, or attenuate the severity, extent or duration of the deleterious effects of a deficiency, or anticipated deficiency, in the corresponding natural polypeptide.

The administration of the agents including polypeptide and nucleic acid compositions of the invention may be for either "prophylactic" or "therapeutic" purpose. When provided prophylactically, the agents are provided in advance of any symptom. The prophylactic administration of the agent serves to prevent or ameliorate any subsequent deleterious effects of the deficiency, or anticipated deficiency in the corresponding natural

polypeptide. When provided therapeutically, the agent is provided at (or shortly after) the onset of a symptom of the deficiency or anticipated deficiency. The agent of the present invention may, thus, be provided either prior to the anticipated deficiency (so as to attenuate the anticipated severity, duration or extent of disease symptoms) or after the deficiency, and its resultant symptoms have manifested themselves.

Also envisioned are therapies based upon vectors and viral particles, such as viral vectors and viral particles containing nucleic acid sequences coding for the polypeptides described herein. These molecules, developed so that they do not provoke a pathological effect, will produce the encoded polypeptides of the invention.

Factor VIII Preparations

The isolation and purification of porcine and human plasma-derived factor VIII and human recombinant factor VIII have been described in the literature. See, e.g., Fulcher, C. A., and T. S. Zimmerman, 79 Proc. Nat'l. Acad. Sci. U.S.A. 1648-1652 (1982); Toole, J. J., et al., 312 Nature 342-347 (1984) (Genetics Institute); Gitschier, J., et al., 312 Nature 326-330 (1984) (Genentech); Wood, W. I., et al., 312 Nature 330-337 (1984) (Genentech); Vehar, G. A., et al., 312 Nature 337-342 (1984) (Genentech); Fass, D. N., et al., 59 Blood 594 (1982); Toole, J. J., et al., 83 Proc. Nat'l. Acad. Sci. U.S.A. 5939-5942 (1986); Boedeker, B.G., Semin. Thromb. Hemost. 27(4):385-94 (Aug. 2001). Two preparations of full-length recombinant factor VIII which were licensed for use in humans in the early 1990s are described, e.g., in Schwartz RS, et al., N Engl J Med 323:1800-5 (1990); Lusher JM, et al., N Engl J Med 328:453-9 (1993); Bray GL, et al., Blood 83:2428-35 (1994); and White GC II, et al., Thromb. Haemost 77:660-7 (1997).

B-domain deleted Factor VIII, which lacks the B domain of the full-length protein but retains coagulant activity, and which has been licensed for use in humans is described, e.g., in Osterbert T, et al., Pharm Res 14:892-8 (1997); Lusher JM, et al., Blood 96:266a (2000) (abstract); and Almstedt et al., U.S. Pat. No. 5,661,008.

Hybrid human/porcine factor VIII has also been described in the literature. See, e.g., U.S. Patent 6,180,371.

The classic definition of factor VIII is that substance present in normal blood plasma that corrects the clotting defect in plasma derived from individuals with hemophilia A. As used herein, factor VIII refers to a molecule which has the procoagulant properties of plasma-derived factor VIII or activated factor VIII. Thus, the term factor VIII, as used

herein, includes a modified or truncated form of natural or recombinant factor VIII which retains the procoagulant properties of factor VIII or activated factor VIII. Thus, factor VIII, as used herein, includes the uncleaved precursor factor VIII molecule, as well as Factor VIII in various proteolytically processed or otherwise truncated forms known to those skilled in the art, wherein the various forms of Factor VIII possess procoagulant activity. Examples of factor VIII polypeptides are those active factor VIII fragments and factor VIII derivatives disclosed in Andersson et al., U.S. Pat. No. 4,749,780; Andersson et al., U.S. Pat. No. 4,877,614; Toole et al., U.S. Pat. No. 4,757,006; Toole, U.S. Pat. No. 4,868,112; Almstedt et al., U.S. Pat. No. 5,661,008, all of which are incorporated herein by reference. The Factor VIII described in Almstedt et al. is made up of amino acids 1 to 743 and 1649 through 2332 of human factor VIII. This polypeptide sequence is commercially known as rFVIII-SQ or REFACTO [r]. See also, Lind et al., Euro. J. Biochem., 232:19-27 (1995). Other forms of truncated FVIII can also be constructed in which the B-domain is generally deleted. In the Almstedt et al. Factor VIII, the amino acids of the heavy chain, containing amino acids 1 through 740 of human Factor VIII and having a molecular weight of approximately 90 kD are connected to the amino acids of the light chain, containing amino acids 1649 to 2332 of human Factor VIII and having a molecular weight of approximately 80 kD. The heavy and light chains are connected by a linker peptide of from 2 to 15 amino acids, for example a linker comprising lysine or arginine residues, or alternatively, linked by a metal ion bond. These other linkers and different sized linkers could be used. See, also, Pipe and Kaufmann (109) for another Factor VIII variant which was genetically engineered by deletion of residues 794-1689 so that the A2 domain is covalently linked to the light chain. Missense mutations at thrombin and activated protein C inactivation cleavage sites provide resistance to proteolysis, resulting in a single-chain protein that has maximal activity after a single cleavage after arginine-372.

A human factor VIII cDNA nucleotide and predicted amino acid sequences are shown in US Patent No. 6,180,371. Factor VIII is synthesized as an approximately 300 kDa single chain protein with internal sequence homology that defines the "domain" sequence NH₂-A1-A2-B-A3-C1-C2-COOH. In US Patent No. 6,180,371, factor VIII domains include the following amino acid residues, when the sequences are aligned with the human amino acid sequence set forth in that patent: A1, residues Ala1-Arg372; A2, residues Ser373-Arg740; B, residues Ser741-Arg1648; A3, residues Ser1690-Ile2032; C1, residues Arg2033-Asn2172; C2, residues Ser2173-Tyr2332. The A3-C1-C2 sequence includes residues

Ser1690-Tyr2332. The remaining sequence, residues Glu1649-Arg1689, is usually referred to as the factor VIII light chain activation peptide. Factor VIII is proteolytically activated by thrombin or factor Xa, which dissociates it from von Willebrand factor, forming factor VIIIa, which has procoagulant function. The biological function of factor VIIIa is to increase the catalytic efficiency of factor IXa toward factor X activation by several orders of magnitude. Thrombin-activated factor VIIIa is a 160 kDa A1/A2/A3-C1-C2 heterotrimer that forms a complex with factor IXa and factor X on the surface of platelets or monocytes or on other surfaces.

The heavy chain of factor VIII contains the A1 and A2 domains and may also contain part or all of the B domain. (The heavy chain of B-domain deleted factor VIII contains two domains, A1 and A2, and may contain a small part of the B-domain.) The light chain of factor VIII contains three domains, A3, C1, and C2.

Factor VIII Pharmaceutical Compositions

Pharmaceutical compositions containing disulfide-stabilized factor VIII, alone or in combination with appropriate pharmaceutical stabilization compounds, delivery vehicles, and/or carrier vehicles, may be prepared according to known methods, such as those described in Remington's Pharmaceutical Sciences by E. W. Martin, incorporated herein by reference. Pharmaceutical compositions may contain factor VIII polypeptide, nucleic acid coding for factor VIII, or the like.

In one preferred embodiment, the preferred carriers or delivery vehicles for intravenous infusion are physiological saline or phosphate buffered saline that may include sugars.

In another preferred embodiment, suitable stabilization compounds, delivery vehicles, and carrier vehicles include but are not limited to other human or animal proteins such as albumin.

Phospholipid vesicles or liposomal suspensions are also preferred as pharmaceutically acceptable carriers or delivery vehicles. These can be prepared according to methods known to those skilled in the art and can contain, for example, phosphatidylserine/-phosphatidylcholine or other compositions of phospholipids or detergents that together impart a negative charge to the surface, since factor VIII binds to negatively charged phospholipid membranes. Liposomes may be prepared by dissolving appropriate lipid(s) (such as stearyl phosphatidylethanolamine, stearyl phosphatidylcholine, arachadoylphosphatidyl choline,

and cholesterol) in an inorganic solvent that is then evaporated, leaving behind a thin film of dried lipid on the surface of the container. An aqueous solution of the factor VIII is then introduced into the container. The solution is mixed to free lipid material from the sides of the container and to disperse lipid aggregates, thereby forming the liposomal suspension.

The factor VIII can be combined with other suitable stabilization compounds, delivery vehicles, and/or carrier vehicles, including vitamin K-dependent clotting factors, tissue factor, von Willebrand factor (vWf) or a fragment of vWF that contains the factor VIII binding site, and polysaccharides such as sucrose.

Factor VIII can be stored bound to vWf to increase the half-life and shelf-life of the molecule. Additionally, lyophilization of factor VIII can improve the yields of active molecules in the presence of vWf. Methods for storage of factor VIII include: lyophilization of factor VIII in a partially-purified state (as a factor VIII "concentrate" that is infused without further purification), and immunoaffinity-purification of factor VIII and lyophilization in the presence of albumin, which stabilizes the factor VIII. Factor VIII can also be prepared by a process that uses sucrose as a stabilizer in the final container in the place of albumin. It is preferred that Factor VIII be prepared by a process that doesn't include any plasma or plasma proteins. (See, e.g., Boedeker (111) and Cho et al., U.S. Pat. No. 6,358,703 B1).

Additionally, factor VIII has been indefinitely stable at 40° C in 0.6M NaCl, 20 mM MES, and 5 mM CaCl₂ at pH 6.0 and also can be stored frozen in these buffers and thawed with minimal loss of activity.

Methods of Treatment

Factor VIII is used to prevent, treat or ameliorate uncontrolled bleeding due to factor VIII deficiency (e.g., intraarticular, intracranial, or gastrointestinal hemorrhage) in subjects such as hemophiliacs with and without inhibitory antibodies and patients with acquired factor VIII deficiency due to the development of inhibitory antibodies (51). The preferred subjects are mammals, most preferably humans. The active materials are preferably administered intravenously.

"Factor VIII deficiency," as used herein, includes deficiency in clotting activity caused by production of defective factor VIII, by inadequate or no production of factor VIII, or by partial or total inhibition of factor VIII by inhibitors. Hemophilia A is a

type of factor VIII deficiency resulting from a defect in an X-linked gene and the absence or deficiency of the factor VIII protein it encodes.

Additionally, factor VIII can be administered by transplant of cells genetically engineered to produce the factor VIII or by implantation of a device containing such cells, as described above.

In a preferred embodiment, pharmaceutical compositions of factor VIII alone or in combination with stabilizers, delivery vehicles, and/or carriers are infused into patients intravenously.

The treatment dosages of factor VIII composition that must be administered to a patient in need of such treatment will vary depending on the severity of the factor VIII deficiency. Generally, dosage level is adjusted in frequency, duration, and units in keeping with the severity and duration of each patient's bleeding episode. Accordingly, the factor VIII is included in the pharmaceutically acceptable carrier, delivery vehicle, or stabilizer in an amount sufficient to deliver to a patient a therapeutically effective amount of the factor VIII to stop bleeding, as measured by standard clotting assays.

Factor VIII is classically defined as that substance present in normal blood plasma that corrects the clotting defect in plasma derived from individuals with hemophilia A. The coagulant activity *in vitro* of purified and partially-purified forms of factor VIII is used to calculate the dose of factor VIII for infusions in human patients and is a reliable indicator of activity recovered from patient plasma and of correction of the *in vivo* bleeding defect. See, e.g., Lusher, J. M., et al., New. Engl. J. Med. 328:453-459 (1993); Pittman, D. D., et al., Blood 79:389-397 (1992), and Brinkhous et al., Proc. Natl. Acad. Sci. USA, 82:8752-8755 (1985).

Usually, the desired plasma factor VIII level to be achieved in the patient through administration of the hybrid or hybrid equivalent factor VIII is in the range of 30-100% of the normal plasma level. Typical dosages for treatment of hemorrhage from hemophilia A with Factor VIII are 25-50 units/kg of body weight. One unit = the normal amount of VIII in 1 ml of citrated normal human plasma. See, e.g., Roberts, HR and Hoffman, M. Hemophilia A and Hemophilia B. in Williams Hematology, 6th edition. eds E Beutler, MA Lichtman, BS Coller, TJ Kipps and U Seligson. McGraw-Hill, NY. 2001. In a preferred mode of administration of factor VIII of the invention, which is expected to have increased stability due to the introduction of one or more cysteine residues, the composition is given intravenously at a preferred dosage in the range from about 0.1 to 80 units/kg body

weight, more preferably in a range of 0.5 to 50 units/kg body weight, more preferably in a range of 1.0-50 units/kg body weight, and most preferably at a dosage of 2.0-40 units/kg body weight; the interval frequency is in the range from about 8 to 24 hours (in severely affected hemophiliacs); and the duration of treatment in days is in the range from 1 to 10 days or until the bleeding episode is resolved. See, e.g., Roberts, H. R., and M. R. Jones, "Hemophilia and Related Conditions-Congenital Deficiencies of Prothrombin (Factor II, Factor V, and Factors VII to XII)," Ch. 153, 1453-1474, 1459-1460, in Hematology, Williams, W. J., et al., ed. (1990). Patients with inhibitors may require more factor VIII of the invention, or patients may require less factor VIII of the invention because of its greater stability than human factor VIII. In treatment with factor VIII, the amount of factor VIII infused is defined by the one-stage factor VIII coagulation assay and, in selected instances, *in vivo* recovery is determined by measuring the factor VIII in the patient's plasma after infusion. It is to be understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that the concentration and other ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed invention.

Treatment can take the form of a single intravenous administration of the composition or periodic or continuous administration over an extended period of time, as required. Alternatively, factor VIII can be administered subcutaneously or orally with liposomes in one or several doses at varying intervals of time.

Hybrid animal/human factor VIII of the invention can be used to treat uncontrolled bleeding due to factor VIII deficiency in hemophiliacs who have developed antibodies to human factor VIII. In this case, coagulant activity that is superior to that of natural human or animal factor VIII alone is not necessary. Coagulant activity that is inferior to that of natural human factor VIII (i.e., less than 3,000 units/mg) will be useful if that activity is not neutralized by antibodies in the patient's plasma.

Factor VIII can also be delivered by gene therapy. The general principles for this type of therapy are known to those skilled in the art and have been reviewed in the literature (e.g. 52, 53, 57). Various strategies have been utilized to deliver factors VIII and IX by gene therapy and many of these may be appropriate for delivery of factor VIII that is modified by the addition of engineered disulfide bonds. Following is a summary of the various approaches that could be utilized.

By far the largest volume of experience has been with retroviral vectors. An example of the extant peer-reviewed and published preclinical data using retroviral vectors to treat hemophilia comes from Kay et al (58), who prepared a retroviral vector expressing canine FIX and infused it into the portal vein of hemophilic dogs that had undergone partial hepatectomy. They were able to demonstrate long-term expression of canine FIX (>2 years) but at levels that were far too low to be therapeutic in humans.

Another approach, also for hemophilia B, makes use of an AAV vector. AAV vectors in present use are engineered from a parvovirus, AAV serotype 2, with a small (4.7 kb) single stranded DNA genome. Many individuals are infected with the wild-type virus as children, but infection is not associated with any known illness. The virus is naturally replication-defective, and the engineered vector is completely devoid of viral coding sequences. Preclinical studies by several groups have shown that AAV vectors can direct sustained expression of a transgene introduced into skeletal muscle, liver, or central nervous system (62-64). In the case of FIX, experiments in mice have resulted in expression levels of 250 to 350 ng/mL (5% to 7% of normal circulating levels), whereas similar experiments in hemophilic dogs resulted in levels of 70 to 80 ng/mL (approx. 1.5% of normal levels (65, 66)).

Efforts are also underway to extend the use of a liver-directed AAV approach to FVIII, but the size of the transgene presents a problem in this case, because AAV vectors cannot accommodate inserts above 5 kb and the B domain-deleted FVIII cDNA (without promoter, intron, or viral-inverted terminal repeats) is 4.4 kb. Because of these size constraints, several novel strategies have been devised to allow expression of FVIII from an AAV vector (76, 77, 78).

A different approach that is currently being evaluated for treatment of hemophilia A is ex vivo introduction of a plasmid expressing B-domain-deleted (BDD) FVIII into autologous fibroblasts, which are then reimplanted on the omentum. In this strategy, a skin biopsy from the patient serves as a source of autologous fibroblasts, which are then transfected by electroporation with a plasmid expressing BDD FVIII and a selectable marker. After transfection, FVIII-expressing cells are selected, expanded, and reimplanted on the omentum in a laparoscopic procedure (using on the order of 10^8 to 10^9 cells) (107).

Adenoviral vectors have several attractive features as gene delivery vehicles, including ease of preparation and efficient transduction of the liver after introduction of vector into the peripheral circulation. These characteristics were exploited by Kay et al (80)

to obtain high-level expression of canine FIX in hemophilic dogs as an early proof of principle for this approach. Several important insights about adenoviral vectors have been gained through the work of Connelly and colleagues (83-87), who have explored the use of earlier generation adenoviral vectors as an approach to treating hemophilia A. Using an adenoviral vector expressing B domain-deleted FVIII, these workers were able to demonstrate phenotypic correction of the bleeding diathesis in mice with hemophilia A (87). Levels of expression were initially >2000 mU/mL and, as expected, declined gradually over 9 months to approx. 100 mU/mL.

Lentiviral vectors (101), a newer gene delivery vehicle based on HIV, have also been shown to transduce liver, muscle, and hematopoietic cells and thus could potentially be used for gene therapy for hemophilia. Work published by Kafri et al (102) demonstrated stable expression (22 weeks) of a humanized GFP after direct intraparenchymal injection into liver of a lentiviral vector.

Okoli et al (106) have presented a preliminary report in which FIX plasmid DNA contained within a chitosan DNA nanosphere is embedded within gelatin cubes and fed to mice at a dose of 25g plasmid in a single treatment. Treated mice showed levels of 45 ng/mL (approx. 1% normal plasma levels), although levels gradually declined to undetectable over a 14-day period.

Phase I clinical trials in humans are underway or in late planning stages for retroviral vectors, AAV vectors, transfected plasmids and adenoviral vectors.

As will be obvious to those of skill in the art, similar methods may be used for the administration of entities other than factor VIII such as factor V, prothrombin, factor XII, HGFA (hepatocyte growth factor activator), and PHBP (plasma hyaluronin binding protein).

The following examples illustrate certain embodiments of the present invention, but should not be construed as limiting its scope in any way. Certain modifications and variations will be apparent to those skilled in the art from the teachings of the foregoing disclosure and the following examples, and these are intended to be encompassed by the spirit and scope of the invention.

Example 1- Factor V

In one embodiment of the present invention, one may engineer into recombinant FV mutants a disulfide bond between the A2 and the A1 or A3 domains such that dissociation of the A2 domain is prevented. Neither the x-ray crystal structure nor NMR

structure of FVa is known. However, as noted above, the present invention is not limited to use with such structures and may be applied to homology models.

Accordingly, the computer program MODIP (19), which employs the algorithm of Sowdhamini, was applied to the Pellequer homology model of FVa (20). As noted above, MODIP predicts sites for the introduction of disulfide bridges and provides grades (A, B, C) for each prediction. Grade A sites are those predicted to be most optimal for the establishment of disulfide bridges, while grade B and grade C sites are progressively less ideal.

For the Pellequer FVa model, no grade A sites were predicted at either the A1-A2 or A2-A3 interfaces, a single grade B site was predicted, and several grade C sites were predicted. Of the grade C sites predicted, MODIP indicated five sites to be the most ideal:

His609 – Glu1691	(A2-A3)
Leu238 – Gln590	(A1-A2)
His253 – Asp469	(A1-A2)
Ala257 – Met618	(A1-A2)
Leu283 – Met618	(A1-A2)

It was noted that of these, the pair 609 – 1691 aligned with residues Tyr664 – Thr1826 in Factor VIII.

Visual inspection of the predicted grade B and C sites using computational graphics analysis showed the grade B site to be unusable. Next, a version of the FVa homology model further including a disulfide bridge was constructed for each of the five best grade C sites. This was done using the Xfit computer program, with refinement being provided by the X-PLOR computer program using the Charm22 all atoms force field.

After refinement, the modeled disulfide bonds were analyzed for optimal disulfide geometry. Cys609 – Cys1691 provided the best potential geometry for a disulfide bond in FV with $r_{ss}=2.02 \text{ \AA}$, $\chi_{ss}=80.9^\circ$, and the lowest Van Der Waals gas phase energy of the five sites. The second best site was Leu238-Gln590, with $r_{ss}=2.03 \text{ \AA}$ and $\chi_{ss}=-111.6^\circ$. Thus, these two sites were chosen for initial attempts to create disulfide bonds using site-directed mutagenesis.

Next a plasmid pED-FV containing full-length FV cDNA was obtained. The full-length FV cDNA in the plasmid pED-FV was then removed by digestion with SalI and inserted into a modified pUC119 plasmid. A fragment of the FV cDNA was next created with

PCR using a 5' primer that created a BamHI site at nt4641 (FV cDNA numbering; nt = nucleotide) and a 3' primer that retained the BamHI site at nt6014 while removing the BamHI site at nt5975. The primers used are shown below, where underlining indicates mutation and boldface indicates a codon or restriction site of interest:

5'-primer (4641 site)

5'-CAC**GGATCCT**ACAGATTACATTGAGATCA-3'

3'-primer (5975 removal, retain 6014)

5'-GTCTGGATCCCTGTGATTATGACTTCCTTTTGCATGTCCACCTGA**A**TCCAAG-3'

The pUC119-FV was digested with BamHI (cutting at nt2601, 5975 and 6014 in FV cDNA numbering). The new PCR fragment was inserted between the BamHI sites in pUC119-FV between nt2601 and 6014. These steps resulted in the removal of nt2602 to 4641 (coding sequence for residues 812 to 1491) creating a construct encoding a B-domainless FV designated FVΔB.

This FVΔB gene construct was inserted into the expression vector pcDNA3.1+ from Invitrogen (Carlsbad, CA). Then, using the Stratagene Quikchange PCR mutagenesis kit (La Jolla, CA) and FVΔB, Ser2183 was mutated to Ala (changing codon AGT to GCC) to prevent glycosylation at Asn2181, yielding the mutant 2183A-FVΔB. This mutation was made to avoid FV heterogeneity due to incomplete glycosylation at Asn2181 which gives two species of FV that differ in certain functional properties (25, 26). All subsequent mutations were made using this B-domainless, Ser2183A mutant. In some embodiments, this step maybe eliminated.

At the same time, the Stratagene Quikchange PCR mutagenesis kit was used to place coding for cysteine residues by the addition of four mutagenic primers. The following pairs were made: Leu238Cys:Gln590Cys (Cys238/Cys590), and His609Cys:Glu1691Cys (A2-SS-A3). Variants were also made with additional mutations of Arg506 and Arg679 to Gln (Gln506 or Gln679) (Q506/Cys238/Cys590, Q506-A2-SS-A3 and Q506/Q679-A2-SS-A3). The mutagenesis primers used are shown below, where underlining indicates mutation and boldface indicates a codon or restriction site of interest:

Ser2183 – Ala

Forward primer

5'-CATGGAATCAAGGCTATTACACTTCGCC-3'

Reverse primer

5'-GGCGAAGTGTAATAGGCTTGATTCCATG-3'

Leu238 – Cys

Forward

5'-GGCCAGAATGGCTTCTCCATTC-3'

Reverse

5'-GAATGGAGAAGGCATTCTGGCC-3'

Gln590 – Cys

Forward

5'-GTGGGGACCTGTAATGAAATT-3'

Reverse

5'-AATTTCAATTACAGGTCCCCAC-3'

His609 – Cys

Forward

5'-CTATGGAAAGAGGTGTGGAGGACACC-3'

Reverse

5'-GGTGTCTCACACCTCTTTCCATAG-3'

Glu1691-Cys

Forward

5'-GATCAGGGCCATGTGAGTCCTGGC-3'

Reverse

5'-GCCAGGACTACATGGCCCTGATC-3'

Arg306 – Gln

Forward

5'-CCAAAGAAAACCCCAGAATCTTAAG-3'

Reverse

5'-CTTAAGATTCTGGGTTTTCTTTGG-3'

Arg506 – Gln

Forward

5'-CTGGACAGGCAAGGAATACAG-3'

Reverse

5'-CTGTATTCCTTGCCTGTCCAG-3'

Arg679 – Gln

Forward

5'-CATGGCTACACAGAAAATGCATG-3'

Reverse

5'-CATGCATTTTCTGTGTAGCCATG-3'

Plasmids containing each mutant were purified with the Qiafilter plasmid midiprep kit from Qiagen, linearized and transfected into COS-1 cells using Superfect transfection reagent according to the manufacturer's instructions. More specifically, 1 µg of DNA was incubated in 60 µL volume of DMEM/F12 media with 5 µL of Superfect reagent for ten minutes. Then 350 µL of DMEM/10%FBS/1mM Glutamine was added and this mixture was transferred to COS-1 cells (about 50% confluent) in wells of a 24-well plate and incubated for 3 hours before washing and replenishing with fresh media. Stable clones were selected using 0.8 mg/mL Geneticin (Gibco BRL, Rockville, MD). Serum-free conditioned media containing 0.05 % BSA and 5mM CaCl₂ was collected from COS-1 cells expressing each FV mutant and was precipitated with 16% PEG 6000. Then the pellet was redissolved in HBS (50 mM HEPES, 150 mM NaCl, pH 7.4) containing 5 mM CaCl₂, 2 mM benzamidine, 5 nM PPACK and 1 mM PMSF, dialyzed versus the same buffer and purified using an anti-FV antibody column (24). Fractions containing FV were collected, concentrated and stored in HBS with 0.1 % BSA at -80 C.

FVa was quantified by activity and by ELISA assay after activation by thrombin. ELISA assays used Nunc Maxisorb plates coated with 10 µg/ml sheep-anti-FV from Affinity Biological (Hamilton, Ontario, CA) and blocked with Superblock from Pierce (Rockford, IL) with antigen detection by mouse anti-FV-light-chain monoclonal antibody (V59). FV (40 nM) was activated with thrombin (0.5 nM) in HBS with 0.1 % BSA and 5 mM CaCl₂ at 37 °C for 10 min and activation was stopped by the addition of 1.1 molar equivalent of hirudin. FVa inactivation assays were performed using FVa at 4 nM and APC at 2.5 nM with determination of residual FVa using prothrombinase assays as described (27). Inactivation of FVa was measured as follows. A mixture of 1 nM FVa with 25 µM phospholipid vesicles was made in 50 mM HEPES, pH 7.4, 100 mM NaCl, 0.5% BSA, 5 mM CaCl₂, 0.1 mM MnCl₂ (called Ptase buffer). Inactivation was initiated by the addition of APC. One µL aliquots were removed at time points and added to 40 µL of 1.25 nM factor Xa

with 25 μ M phospholipid vesicles, followed by 10 μ L of 3 μ M prothrombin (final concentrations: 1 nM FXa, 20 pM FVa, 25 μ M phospholipid vesicles and 0.6 μ M prothrombin). After 2.5 min a 15 μ L aliquot of this mixture was quenched by addition to 55 μ L TBS containing 10 mM EDTA, 0.5 % BSA, pH 8.2. Chromogenic substrate CBS 34-47 was added and the amount of thrombin formation was assessed by measuring the change in absorbance at 405 nm.

For some studies, FXa or prothrombin was varied. For Xa titrations, a mix of 3.34 pM FVa/FVai and 41.7 μ M phospholipid vesicles in Ptase buffer was aliquoted in 30 μ L aliquots into wells of 96-well plate (polypropylene, V-well). 10 μ L of Xa was added to each well in the same buffer at various concentrations. At time = 0, 10 μ L of 1.5 μ M prothrombin (FII) was added to all wells (final concentrations = 2 pM FVa, 25 μ M PL vesicles, 5-600 pM Xa, 0.3 μ M FII). At time = 12 min, the Ptase reaction was stopped by removing 15 μ L to a 96-well plate containing 55 μ L TBS containing 0.5% BSA, 10 mM EDTA at pH 8.2. Next, the amount of thrombin formed was measured with the chromogenic substrate CBS 34-47. For prothrombin, 20 μ L of mix containing 125 pM Xa, 1.25 nM FVa/FVai, and 31.25 μ M PL vesicles in Ptase buffer was aliquoted into wells of 96-well V-well plate. At time = 0, 5 μ L FII at varying concentrations (final concentration 100 pM Xa, 1 nM FVa, 25 μ M PL, 25-1500 nM FII) was added. At time = 2:30, the reaction was stopped by removing 15 μ L to 55 μ L EDTA buffer as above. Thrombin was measured as above.

SDS-PAGE was then performed with Novex 4-12 % Bis-Tris gradient gels with MOPS buffer (Invitrogen, Carlsbad, CA). 50 ng protein was loaded per lane. The proteins were then transferred to Millipore PVDF membranes, and immunoblots were developed with monoclonal anti-FV-light chain antibodies, AHV-5112 or V59, and rabbit polyclonal anti-FV-heavy chain antibodies (24). More specifically, membranes were blocked with TBS, 1% Casein, and 2 mM CaCl_2 . Antibodies were diluted in the same buffer. The primary antibody was the respective anti-FV antibody, and the secondary antibody was biotinylated goat anti-mouse IgG or biotinylated donkey anti-rabbit IgG from Pierce. Visualization was then performed with streptavidin-conjugated alkaline phosphatase and 1-step NBT/BCIP substrate (also from Pierce). For the FV species that were produced and purified, yields of pure FV ranged from 5 to 25 μ g/L of conditioned media. Based on silver-stained SDS-PAGE, we estimated the purity of the mutants to range from 70% to 90%.

As is known in the art, the FVa light chain normally gives a doublet on SDS-PAGE due to heterogeneity created by incomplete glycosylation at Asn2181. Mutation of Ser2183 to Ala eliminates this glycosylation site (28). Immunoblots confirmed that all our recombinant FV molecules had an apparent molecular weight of 188 kDa, consistent with deletion of residues 812 to 1491. Immunoblots further confirmed that the wild type recombinant FVa formed a light chain doublet, whereas all other Fva mutants carrying the Z183A mutation had only a single light chain band.

To demonstrate the desired interdomain disulfide bonds in the mutant FV proteins containing two engineered cysteine residues, immunoblots of FVa and APC-treated FVai (where "i" indicates inactivated) were run. Fig. 1 shows schemes representing the primary sequences of FV Δ B, FVa (formed upon thrombin activation), and FVai (inactivated by APC cleavages).

Immunoblots using a polyclonal anti-FV heavy chain antibody demonstrated that introduction of Cys238/Cys590 mutations into FV or Q506-FV did not detectably link the A1 and A2 domains although these species had normal FVa activity, leading us to conclude that no disulfide bond was formed between these cysteines.

If FV mutants containing Cys609 and Cys1691 generate a new disulfide bond between the A2 and A3 domains as depicted in Fig. 1C, it would link the FVa heavy and light chains. In this case, in immunoblots of FVa, the disulfide-bonded species would appear at a molecular weight corresponding to the additive molecular weights of the heavy and light chains, and following APC cleavages at Arg506, Arg306 and Arg679 that normally cause complete FVa inactivation, the light chain of FVai would remain cross-linked to the C-terminal fragment of the A2 domain (A2c, residues 507 to 679).

Indeed such results were obtained. In immunoblots developed with anti-FV light chain antibodies (Fig. 2A), lanes 1 and 2 containing 2183A-FVa and 2183A-FVai both showed a normal light chain at the expected molecular weight (69 kDa), whereas in lane 3, the mutant containing Cys609/Cys1691-FVa showed a predominant band predicted for cross-linked light chain and heavy chain (158 kDa). Thus, FV mutants containing these two Cys residues are justifiably designated "A2-SS-A3".

Lane 4 demonstrated that APC-treated A2-SS-A3-FVai gave a predominant band corresponding to the mobility predicted for the light chain cross-linked to the A2c fragment (92 kDa). A fainter band slightly above this band correlated with a band predicted for heavy chain cleaved at Arg506 but not Arg679, resulting in the fragment 507 to 709 (101

kDa). Lanes 5 and 6 (Fig. 2A) contained Q506-A2-SS-A3-FVa and Q506-A2-SS-A3-FVai. In these species, Arg506 cleavage cannot take place such that in Q506-A2-SS-A3-FVai (lane 6) the light chain remained cross-linked to the entire A2 domain (with or without its small C-terminal tail of residues 680-709). Indeed, the observed higher molecular weight band (lane 6) corresponded to the light chain cross-linked to the A2 domain (130 kDa). Lanes 7 through 12 of Fig. 2A contained samples parallel to those of lanes 1 through 6, which were reduced using DTT. Lanes 7-12 show that, following reduction, the various higher molecular weight cross-linked species disappeared and normal light chain bands appeared, proving that the higher molecular weight light chain-containing species seen in lanes 3-6 (Fig. 2A) were indeed the result of disulfide cross-links between light and heavy chains.

Additional proof for covalent cross-links between FVa heavy and light chains in A2-SS-A3 mutants containing Cys609/Cys1691 came from immunoblot analyses using anti-FV heavy chain antibodies that showed, under non-reducing conditions, the same new bands visualized in immunoblots developed using anti-FV light chain antibodies. For example, in Fig. 2B such immunoblots of A2-SS-A3-FVa and A2-SS-A3-FVai as well as Q506-A2-SS-A3-FVa and Q506-A2-SS-A3-FVai under non-reducing conditions gave bands predicted to represent the same cross-linked species visualized in immunoblots developed using anti-FV light chain antibodies Fig. 2B. Lanes 1 and 5 (Fig. 2B) both showed bands corresponding to light chain cross-linked to heavy chain that co-migrated with that seen in Fig. 2B, lane 3 (157 kDa). Lane 2 in Fig. 2B showed a band corresponding to the light chain cross-linked to the A2c fragment, co-migrating with a band seen in lane 4 of Fig. 2A (102 kDa). Lane 6 in Fig. 2B showed a band corresponding to the light chain cross-linked to the A2 domain, equivalent to a band seen in lane 6 of Fig. 2A (132 kDa).

Finally, free A2-C terminus fragment (24 kDa) and A2 (63 kDa) fragment were not visible in the non-reduced lanes 2 and 6, respectively, but were visible in the reduced lanes 4 and 8, indicating that these fragments were released from the disulfide-linked species upon reduction.

Immunoblot analyses of Q506-A2-SS-A3 FVa and Q506/Q679-A2-SS-A3-FVa showed that there was a small amount of free light chain that was not cross-linked to heavy chain (Fig. 2), indicating that disulfide cross-linking in the A2-SS-A3-FVa mutants was not 100 % complete. Densitometry analysis of these non-reduced immunoblots showed that, on average, about ten percent of the Q506-A2-SS-A3-FVa molecules lacked disulfide cross-links.

As alluded to above, Fig. 1A is a schematic of the primary sequence of FV Δ B with the locations of the different domains indicated. The schematic of Fig. 1b shows activated FV Δ B (FVa), a heterodimer of the N-terminal heavy chain and the C-terminal light chain associated in the presence of Ca²⁺ ions. Arrows indicate sites of cleavage in FVa by APC. The schematic of Fig. 1C shows the cleavage fragments produced upon inactivation of FVa (FVai) by APC, and further shows the sites of the cysteine mutations that did (His609-Glu1691) and did not (Leu238-Gln590) result in disulfide bond formation.

Example 2- Factor VIII

As is known in the art, there are a number of similarities between Factor V and Factor VIII. More specifically, Factors V and VIII have similar gene structures, have highly homologous amino acid sequences and domain structures, are both activated by highly specific cleavages by thrombin, and both are inactivated by limited proteolysis by activated protein C (APC). Accordingly, one may engineer into recombinant FVIII disulfide bonds between the A2 and the A1 or A3 domains using a method similar to that disclosed above concerning FV. As is known in the art, FVIIIa is thermodynamically unstable because the A2 domain can spontaneously disassociate. As shown in Fig. 3, placement of a disulfide bond between the A2 and the A1 or A3 domains of FVIIIa has the advantage of preventing this dissociation.

Like FVa, neither the x-ray crystal nor NMR structure of FVIIIa is known. However, as noted above, the present invention is not limited to use such structures and may be applied to homology models.

As a first step in engineering a disulfide bond between the A2 and the A1 or A3 domains of FVIIIa, the computer program MODIP, which employs the algorithm of Sowdhamini, was applied to the Pemberton et al. (54) homology model of the A domains of FVIIIa. As noted above, MODIP predicts sites for the introduction of disulfide bridges and provides grades (A, B, C) for each prediction. Grade A sites are those predicted to be most optimal for the establishment of disulfide bridges, while grade B and grade C sites are progressively less ideal. For the Pemberton FVIIIa model fifteen sites were predicted:

Grade A:

Met 662 – Asp 1828 (A2-A3)

Grade B:

Ser 268 – Phe 673 (A1-A2)
 Ile 312 – Pro 672 (A1-A2)
 Ser 313 – Ala 644 (A1-A2)
 Met 662 – Lys 1827 (A2-A3)
 Tyr 664 – Thr 1826 (A2-A3)

Grade C:

Pro 264 – Gln 645 (A1-A2)
 Arg 282 – Thr 522 (A1-A2)
 Ser 285 – Phe 673 (A1-A2)
 His 311 – Phe 673 (A1-A2)
 Ser 314 – Ala 644 (A1-A2)
 Ser 314 – Gln 645 (A1-A2)
 Val 663 – Glu 1829 (A2-A3)
 Asn 694 – Pro 1980 (A2-A3)
 Ser 695 – Glu 1844 (A2-A3)

Of these, the pair Tyr 664 – Thr 1826 was noticed to be in a position homologous to the pair His609 – Glu1691 in FVa. As noted above, a disulfide bridge may be successfully engineered into FV by placing coding for cysteine residues at positions 609 and 1691.

Similar to the method described above for FV, visual inspection of these pairs was performed using computational graphics analysis. As a result of this analysis, three of the proposed pairs were chosen for further investigation: Met 662 – Asp 1828, Tyr 664 – Thr 1826 and Ser 313 – Ala 644. For each of these three sites, a version of the FVIIIa model further including a disulfide bridge at the appropriate location was constructed using the Xfit computer program, with refinement being provided by the X-PLOR computer program using the Charm22 all atoms force field. After refinement, the modeled disulfide bonds were ranked in the order given above with Cys 662 – Cys 1828 providing the best potential geometry for a disulfide bond. It was chosen to make this mutant and the mutant Cys 664 – Cys 1826 in recombinant factor VIII in a manner analogous to that described above with reference to FV.

A FVIII expression plasmid (p25D) was obtained from Bayer Corporation. This plasmid expresses B-domain deleted FVIII in which residues 744 to 1637 from the B domain are deleted.

Next, using the Stratagene Quikchange PCR mutagenesis and the mutant FVIII, two cysteine residues were inserted to permit the creation of a disulfide bond by the addition of four mutagenic primers at the same time. The following two pairs were made: Met662Cys:Asp1828Cys and Tyr664Cys:Thr1826Cys. The mutagenesis primers used are shown below, where underlining indicates mutation and boldface indicates a codon or restriction site of interest:

Met662 – Cys

Forward

5'-CCTTCAAACACAAATGCGTCTATGAAGACACACTCACC-3'

Reverse

5'-GGTGAGTGTGTCTTCATAGACCGCATTTGTGTTTGAAGG-3'

Asp1828 – Cys

Forward

5'-GGCACCCACTAAATTGTGAGTTTGACTGCAAAGC-3'

Reverse

5'-GCTTTGCAGTCAAAC**TCA**CATTTAGTGGGTGCC-3'

Tyr664 – Cys

Forward

5'-CACAAAATGGTCTTGTGAAGACACACTCACCC-3'

Reverse

5'-GGGTGAGTGTGTCTTCACAGAGGATTTTGTG-3'

Thr1826 – Cys

Forward

5'-CATATGGCACCC**TG**TAAAGATGAGTTTGACTGC-3'

Reverse

3'-GCAGTC**AA**ACTCATCTTTACAGGGTGCCATATG-3'

The Tyr664-Cys reverse primer shown above was the actual sequence used but the actual FVIII gene sequence at nucleotides 22 and 23 should be CC rather than GG.

But the forward primer has the correct sequence and the correct sequence was selected for the final C664 mutant by DNA sequencing of the selected clones.

Fig. 4 is a schematic showing the expected action of APC upon mutant FVIII containing a disulfide bridge between sites Met 662 - Asp 1828 or Tyr 664 - Thr 1826.

In some embodiments, variants may be made which additionally contain mutations and/or deletions of APC cleavage sites Arg 336 and/or Arg 562 in FVIII. Such additional mutations, as described in Kaufman and Pipe (109) and in U.S. Patents 5,422,260, 5,250,421, 5,198,349 (incorporated herein by reference), add additional stability to FVIII by making it more resistant to inactivation.

The nucleic acids encoding Factor VIII mutants may also be modified to contain an increased number of preferred codons for human genes as described, e.g., in Seed et al., U.S. Patent No. 6,114,148.

Transient expression of wildtype and mutant p25D plasmid was tested in COS-1 cells, K293 cells and BHK-21 cells using Superfect reagent and Effectene reagent, both from Qiagen. The Effectene reagent in K293 cells gave the best results. Yields of recombinant FVIII ranged from 10 to 100 mU/mL of conditioned media according to APTT activity assays and ELISA (Immubind FVIII ELISA, American Diagnostica). Conditioned media was collected from transient transfections in 100 mm dishes in DMEM/F12 media with 2 % FBS and the media was concentrated 15-fold and dialyzed into HEPES buffered saline/5 mM CaCl₂/ 0.1 mM MnCl₂, pH 7.4. Mock transfection media was treated in the same manner and used as a negative control.

Antigen concentration of recombinant FVIII was determined using the Immubind FVIII ELISA kit from American Diagnostica. The standard curve used was the purified FVIII concentrate provided with the kit (1 unit = the FVIII contained in 1 mL of plasma). Activity was determined with an APTT assay with FVIII deficient plasma and the APTT reagent Platelin LS as follows: 50 μ L of FVIII deficient plasma (FVIII_{id}P, George King Biomedical) was mixed with 50 μ L Platelin LS (Organon Teknika) and incubated at 37 °C for three minutes. 5 μ L of a FVIII sample was then added, immediately followed by 50 μ L of HEPES buffered saline (0.15 M NaCl) with 0.5% BSA and 25 mM CaCl₂. Clotting time was measured in the Diagnostica Stago ST4 coagulometer. A FVIII standard curve was made using pooled normal human plasma (George King Biomedical), which is defined to contain 1.0 unit/mL of FVIII. The APTT assay was sensitive to very low levels of FVIII (< 0.005 U/mL).

Using these measures of antigen and activity, relative specific activity of the three proteins was calculated (units (U) activity/units (U) antigen). The wildtype FVIII (B domain-deleted) had a relative specific activity of 0.83. C662-C1828-FVIII had a relative specific activity of 3.53 and C664-C1828-FVIII had a relative specific activity of 3.40.

The stability of thrombin-activated FVIIIa over time was followed using a protocol described by Pipe et al (110) with some modification, in which FVIIIa at a concentration of about 500 mU/mL was generated by the addition of thrombin which was then inactivated with a slight excess of hirudin. Subsequently, aliquots of this mix were removed over time and immediately assayed for FVIIIa activity in the APTT assay as described above. Fig. 5 shows the results of this assay with recombinant wildtype FVIIIa and two recombinant mutants. The two double-cysteine mutants are much more stable over time than wildtype FVIIIa (as reflected in a shorter clotting time). The mock transfection control conditioned media showed essentially no coagulant activity in this assay and no change in activity over the time course (data not shown).

The FVIII mutant produced may be stably transfected into cells. The cells can be grown (or cultured) to permit expression of the FVIII mutants. The FVIII mutant produced may be isolated and purified. In a manner described above with reference to FV, immunoblots may be performed to confirm the lack of the majority of the B domain (if appropriate) and the presence of the engineered disulfide bonds.

Example 3- Porcine-Human Hybrid Factor VIII

There exists in the art hybrid factor VIII molecules whose amino acid sequence derives from both human and non-human-animal ("non-human") factor VIII coding sequences. Examples of such molecules may be found, for example in U.S. Patent 6,180,371, incorporated herein by reference. According to the present invention, non-human/human hybrid factor VIII containing a disulfide bond between the hybrid's A2 and A1 or A3 domains may be created. Like the above example, such a disulfide bond prevents dissociation of the A2 domain.

The creation of such hybrid molecules is largely analogous to the procedure described above for non-hybrid FVIII. Firstly, a homology model of hybrid FVIIIa, for example, one comprised of a non-human A2 domain and a heterodimer of des-A2 human factor VIIIa, may be obtained or created. Alternately, an x-ray crystal structure may be obtained or created if such a structure exists or is capable of being created. The MODIP

computer program may next be run on the model or structure so as to receive from the program suggestions of sites for the formation of a disulfide bridge between the A2 and A1 or A3 domains of the hybrid. Alternately, predictive methods may be used as described above.

Next, visual inspection of one or more of the suggested sites may be preformed using computational graphics analysis. As a result of this analysis, a number of proposed sites may be chosen for further investigation. For each of these sites, a version of the hybrid FVIIIa model further including a disulfide bridge at the appropriate location may be constructed using the Xfit computer program, with refinement being provided by the X-PLOR computer program using the Charm22 all atoms force field. After refinement, the modeled disulfide bonds may be ranked based on quality of potential geometry for a disulfide bond. A number of the suggested sites may then be chosen for attempted creation of mutant hybrid FVIII in a manner analogous to that described in reference to FV and non-hybrid FVIII above.

Example 4- Prothrombin

As noted above, in the presence of thrombomodulin and phosphatidylserine/phosphatidylcholine phospholipid vesicles (PCPS), meizothrombin, as well as meizothrombin (des F1), are better activators of protein C than thrombin.

According to the present invention, a mutant prothrombin may be created which includes a disulfide bond to stabilize prothrombin's meizothrombin (des F1) form, and to prevent the conversion of meizothrombin (des F1) to thrombin. Such a stable meizothrombin (des-F1) has potential application, for example, as an anticoagulant. It was decided to achieve this stabilization by placement of a disulfide bond between the Kringle 2 and protease domains of prothrombin as shown in Fig. 5.

First, the computer program MODIP was applied to the X-ray crystal structure of a human thrombin complex of alpha-thrombin and fragment 2 (55), and the X-ray crystal structure of bovine meizothrombin (des F1) (108) resulting in the following predicted sites in human prothrombin:

Grade B:

Asp261-Arg443	(KR2-protease)
His205-Lys572	(KR2-protease)

Grade C:

Asp261-Lys567 (KR2-protease)

Next, visual inspection of one or more of the suggested sites may be performed using computational graphics analysis. As a result of this analysis, a number of proposed sites may be chosen for further investigation. For each of these three sites, a meizothrombin (des F1) homology model including a disulfide bridge at the appropriate location may be constructed using the Xfit computer program, with refinement being provided by the X-PLOR computer program using the Charm22 all atoms force field. After refinement, the modeled disulfide bonds may be ranked based on quality of potential geometry for a disulfide bond. A number of the suggested sites, or sites that have not yet been identified, may then be chosen for attempted creation of mutant prothrombin in a manner analogous to that described in reference to FV and non-hybrid FVIII above.

Example 5- Factor XII, HGFA, and PHBP

As noted above, at least two forms of activated factor XII (FXIIa) exist, α FXIIa and FXIIa fragment. As also noted above, the FXIIa fragment has the bulk of its N-terminal heavy chain fragment no longer associated such that it no longer binds to surfaces but it is still catalytically active. According to the present invention, a disulfide bond can be placed that crosslinks this N-terminal heavy chain fragment to the remainder of the molecule, causing it to retain its surface binding characteristics. It is expected that such a mutant stabilized FXII could find pharmaceutical application as a coagulant.

A second FXII-like polypeptide is HGFA. HGFA activates hepatocyte growth factor (HGF) within injured tissues, where HGF plays roles in tissue repair. As noted above, cleavage of HGFA by kallikrein at Arg372 results in release of the N-terminal heavy chain, which, as in FXII, is involved in surface binding. According to the present invention, a disulfide bond can be placed to prevent the release of the N-terminal heavy chain. It is suspected that a mutant HGFA so stabilized could be used pharmaceutically to aid in tissue repair.

A third FXII-like polypeptide is PHBP. As noted above, PHBP activates FVII, uPA, and tPA and has a structure homologous to HGFA. According to the present invention, a disulfide bond could be placed to prevent the release of the N-terminal heavy chain in

PHBP. It is suspected that a mutant PHBP so stabilized could be used pharmaceutically to promote clotting via activation of FVII, uPA, and/or tPA.

No x-ray crystal or NMR structure exists for Factor XII, HGFA, or PHBP. However, homology models for these molecules, such as ones based on the x-ray crystal structure of urokinase, may be created or obtained. Using such homology models, mutants may be created in a manner analogous to that described above with reference to, for example, FV and FVIII.

Example 6- Other Clotting Factors

As is known in the art, several plasma factors other than factors V and VIII are synthesized as a single polypeptide chain, contain multiple independently folded domains, and are subject to limited proteolysis that may result in separation of domains due to dissociation. As noted above, the methods described herein may be used in all cases where one wishes to place a disulfide bond between two domains of a polypeptide. Accordingly, it should be apparent to those in the art that the methods described herein may be applied to a multitude of polypeptides, including many of the human and non-human clotting factors.

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Every reference cited here and throughout the application is hereby incorporated by reference in its entirety.

Ramifications and Scope

Changes may be made in the nature, composition, operation and arrangement of the various elements, steps and procedures described herein without departing from the spirit and scope of the invention as defined in the following claims. Modifications of the above described modes for carrying out the invention that are obvious to those of skill in the fields of genetic engineering, virology, hematology, medicine, and related fields are intended to be within the scope of the following claims.

CLAIMS

1. A method of stabilizing a polypeptide which is the product of a single gene in nature by introducing one or more cysteines comprising the steps of:

(a) obtaining or creating a three-dimensional structure of said polypeptide;

(b) predicting one or more sites for the introduction of one or more cysteines based on said structure; and

(c) creating one or more mutants of said polypeptide by introducing one or more cysteines at one or more of said predicted sites;

wherein the introduction of said one or more cysteines permits the formation of at least one intramolecular, interdomain disulfide bridge which increases the stability of the mutant polypeptide as compared to that of the polypeptide which does not contain said introduced one or more cysteines.

2. A method of stabilizing a polypeptide which is the product of a single gene in nature by introducing one or more cysteines comprising the steps of:

(a) obtaining or creating a three-dimensional structure based on homology modeling of said polypeptide;

(b) predicting one or more sites for the introduction of one or more cysteines based on the three dimensional structure; and

(c) creating one or more mutants of said polypeptide by introducing one or more cysteines at one or more of said predicted sites;

wherein the introduction of said one or more cysteines permits the formation of at least one intramolecular, interdomain disulfide bridge which increases the stability of the mutant polypeptide as compared to that of the polypeptide which does not contain said introduced one or more cysteines.

3. The method of claim 1 or 2 wherein said polypeptide is a plasma protein.

4. The method of claim 1 or 2 wherein said polypeptide is selected from the group consisting of Factor VIII, Factor V, prothrombin, Factor XII, hepatocyte growth factor activator, and plasma hyaluronan binding protein.

5. The method of claim 4 wherein said polypeptide is Factor V or Factor VIII.

6. A polypeptide which is the product of a single gene in nature which has been mutated by introducing at least one cysteine, wherein the introduction of said cysteine permits the formation of at least one intramolecular, interdomain disulfide bridge with another cysteine, which increases the stability of the mutant polypeptide as compared to that of the polypeptide which does not contain said introduced cysteine.

7. A composition comprising a polypeptide of claim 6.

8. A pharmaceutical composition comprising a polypeptide of claim 6 and a pharmaceutically acceptable carrier.

9. A nucleic acid coding for a polypeptide of claim 6.

10. A vector comprising the nucleic acid of claim 6.

11. A host cell comprising a nucleic acid of claim 6.

12. A mutant Factor VIII which comprises at least one pair of cysteines located at residues selected from the group consisting of Met 662 – Asp 1828, Ser 268 – Phe 673, Ile 312 – Pro 672, Ser 313 – Ala 644, Met 662 – Lys 1827, Tyr 664 – Thr 1826, Pro 264 – Gln 645, Arg 282 – Thr 522, Ser 285 – Phe 673, His 311 – Phe 673, Ser 314 – Ala 644, Ser 314 – Gln 645, Val 663 – Glu 1829, Asn 694 – Pro 1980, and Ser 695 – Glu 1844.

13. The mutant Factor VIII of claim 12 wherein the pair of cysteines is located at residues selected from the group consisting of Met 662 – Asp 1828 and Tyr 664 – Thr 1826.

14. A composition comprising a mutant Factor VIII of claim 12, 13 or 19.

15. A pharmaceutical composition comprising a mutant Factor VIII of claim 12, 13 or 19 and a pharmaceutically acceptable carrier.

16. A nucleic acid coding for a mutant Factor VIII according to claim 12, 13 or 19.

17. A vector comprising the nucleic acid of claim 16.

18. A host cell comprising a nucleic acid of claim 16.

19. A mutant Factor VIII comprising: a non-human animal or human A2 domain containing an introduced cysteine; and a human Factor VIII comprising the domain A1 and the A3-C1-C2 domains with an introduced cysteine; wherein the cysteines are chosen to permit formation of a disulfide bond.

20. A factor V mutant which comprises at least one pair of cysteines located at residues selected from the group consisting of His609 – Glu1691, Leu238 – Gln590, His253 – Asp469, Ala257 – Met618, and Leu283 – Met618.

21. A factor V mutant of claim 20 wherein the pair of cysteines is located at residues His609-Glu1691.

22. A composition comprising a mutant Factor V of claim 20 or 21.

23. A pharmaceutical composition comprising a mutant Factor V of claim 20 or 21 and a pharmaceutically acceptable carrier.

24. A nucleic acid coding for a mutant Factor V of claim 20 or 21.

25. A vector comprising the nucleic acid of claim 24.

26. A host cell comprising a nucleic acid of claim 24.

27. A method of treating increased risk of bleeding in a subject with a pharmaceutical composition comprising an effective amount of factor VIII mutant of claim 4, 5, 6, 12 or 13.

28. A method of treating increased risk of bleeding in a subject with a pharmaceutical composition comprising an effective amount of factor V mutant of claim 4, 5, 6, 20 or 21.

29. A method for enhancing blood coagulation in a subject comprising administering a pharmaceutical composition comprising an effective amount of a factor VIII mutant of claim 4, 5, 6, 12, 13 or 19.

30. A method for enhancing blood coagulation in a subject comprising administering a pharmaceutical composition comprising an effective amount of factor V mutant of claim 20 or 21.

31. A method for treating hemophilia in a subject comprising administering a pharmaceutical composition comprising an effective amount of a Factor VIII mutant of claim 4, 5, 6, 12, 13 or 19.

32. A method for treating hemophilia in a subject comprising administering a pharmaceutical composition comprising an effective amount of a Factor V mutant of claims 20 or 21.
33. A mutant prothrombin comprising one or more introduced cysteines wherein at least one of the introduced cysteines is capable of forming a disulfide bond with another cysteine residue, wherein said disulfide bond is formed between the Kringle 2 and protease domains of said prothrombin mutant.
34. A mutant prothrombin which comprises at least one pair of cysteines located at residues selected from the group consisting of Asp 261-Arg 443, Asp 261-Lys 567, and His 205-Lys 572.
35. A composition comprising a mutant prothrombin of claim 33 or 34.
36. A pharmaceutical composition comprising a mutant prothrombin of claim 33 or 34 and a pharmaceutically acceptable carrier.
37. A nucleic acid coding for a mutant prothrombin of claim 33 or 34.
38. A vector comprising the nucleic acid of claim 37.
39. A host cell comprising a nucleic acid of claim 37.
40. A method for providing antithrombotic activity in a subject comprising administering a pharmaceutical composition comprising an effective amount of a prothrombin mutant of claim 33 or 34.
41. A composition comprising a nucleic acid of claim 9.
42. A composition comprising a nucleic acid of claim 16.
43. A composition comprising a nucleic acid of claim 24.
44. A composition comprising a nucleic acid of claim 37.
45. A method of treating Factor VIII deficiency in a mammal, comprising:
(a) providing a nucleic acid, vector or virion comprising a nucleotide sequence encoding a Factor VIII mutant of any one of claims 12, 13 or 19 operably linked to expression control elements; and
(b) administering said nucleic acid, vector or virion to a mammal under

conditions that result in the expression of Factor VIII mutant at a level that provides a therapeutic effect in said mammal.

46. A method of treating Factor VIII deficiency in a mammal, said method comprising:

(a) providing a first nucleic acid, vector or virion comprising a nucleotide sequence encoding the light chain of a Factor VIII mutant of any one of claims 12, 13 or 19 operably linked to expression control elements; and

(b) providing a second nucleic acid, vector or virion comprising a nucleotide sequence encoding the heavy chain of a Factor VIII mutant of any one of claims 12, 13 or 19 operably linked to expression control elements; and

(c) administering said first and second nucleic acid, vector or virion to a mammal under conditions that result in the expression of Factor VIII mutant at a level that provides a therapeutic effect in said mammal.

47. A method of treating Factor VIII deficiency in a mammal, comprising delivering a host cell comprising a nucleotide sequence encoding Factor VIII to a mammal said method comprising:

(a) providing a host cell comprising a nucleotide sequence encoding a Factor VIII mutant of any one of claims 12, 13 or 19 operably linked to expression control elements; and

(b) administering said host cell to a mammal under conditions that result in the expression of Factor VIII mutant protein at a level that provides a therapeutic effect in said mammal.

48. A method of treating Factor VIII deficiency in a mammal, comprising delivering host cells comprising a nucleotide sequences encoding Factor VIII to a mammal, said method comprising:

(a) providing a host cell comprising (i) a first nucleotide sequence encoding the light chain of Factor VIII mutant of any one of claims 12, 13 or 19 operably linked to expression control elements; and (ii) a second nucleotide sequence encoding the heavy chain of Factor VIII mutant of any one of claims 12, 13 or 19 operably linked to expression control elements; and

(b) administering said host cell to a mammal under conditions that result in the

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expression of Factor VIII mutant protein at a level that provides a therapeutic effect in said mammal.

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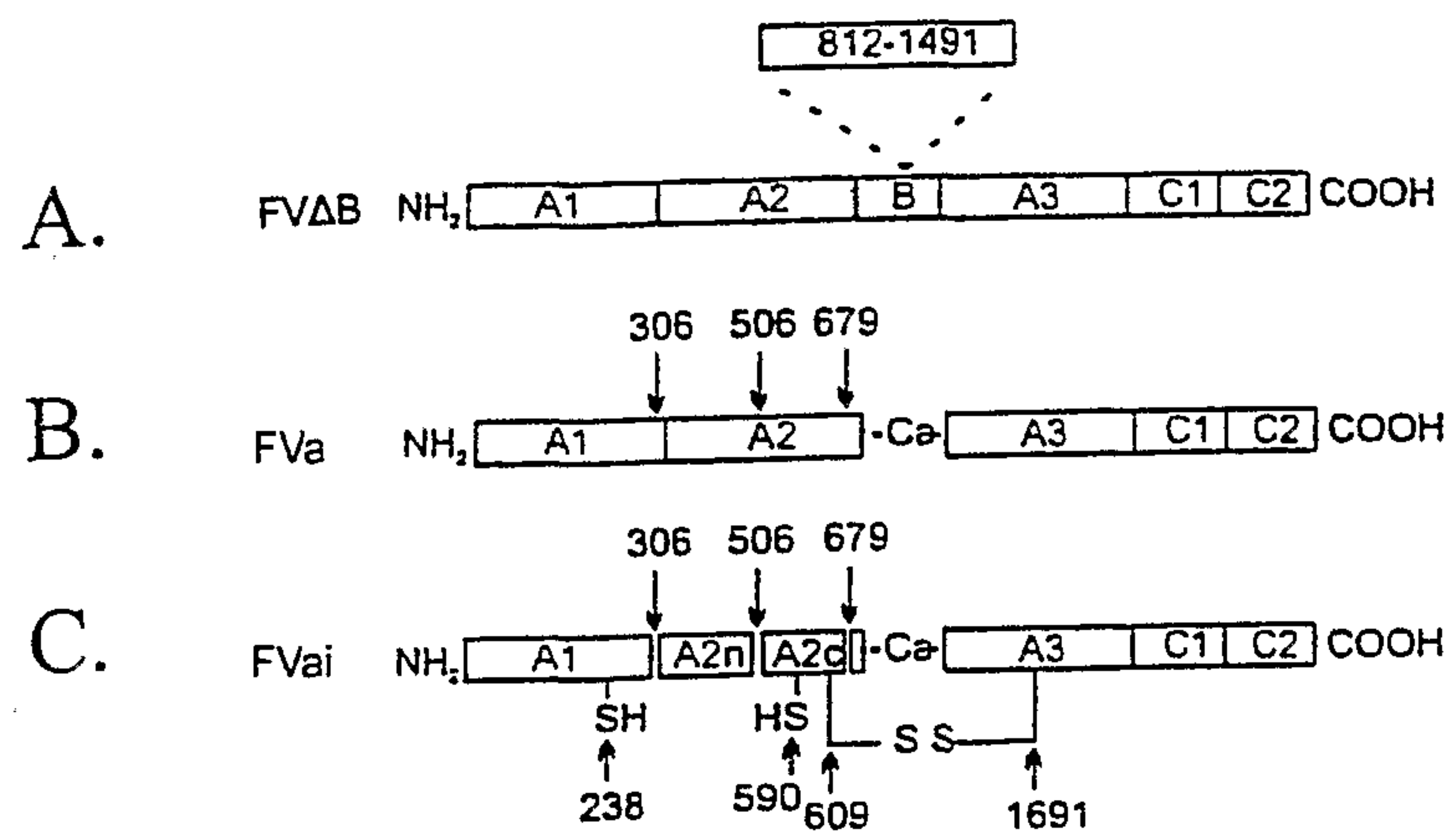


FIG. 1

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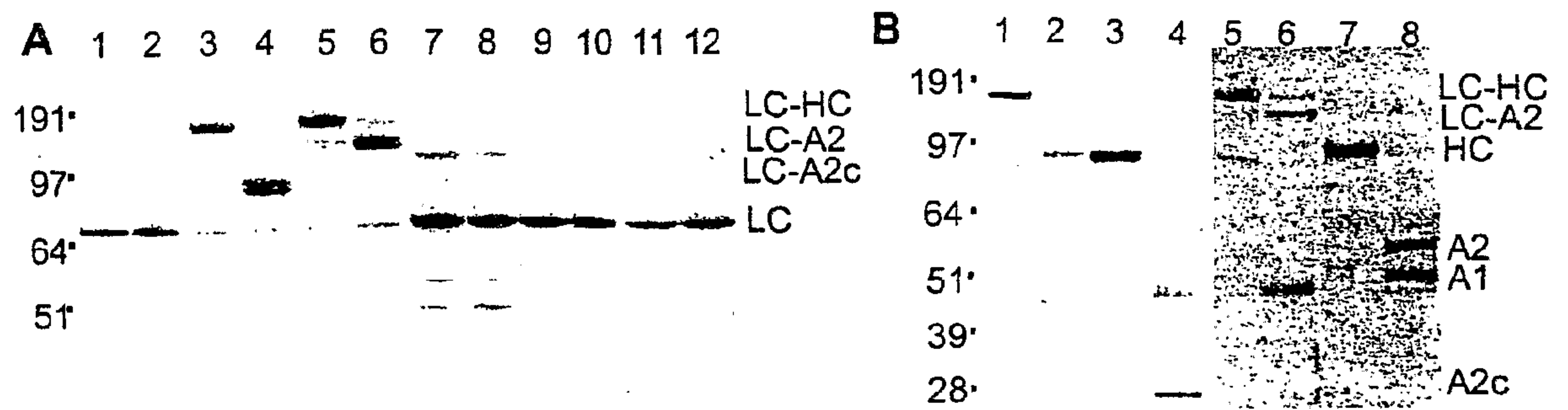


FIG. 2

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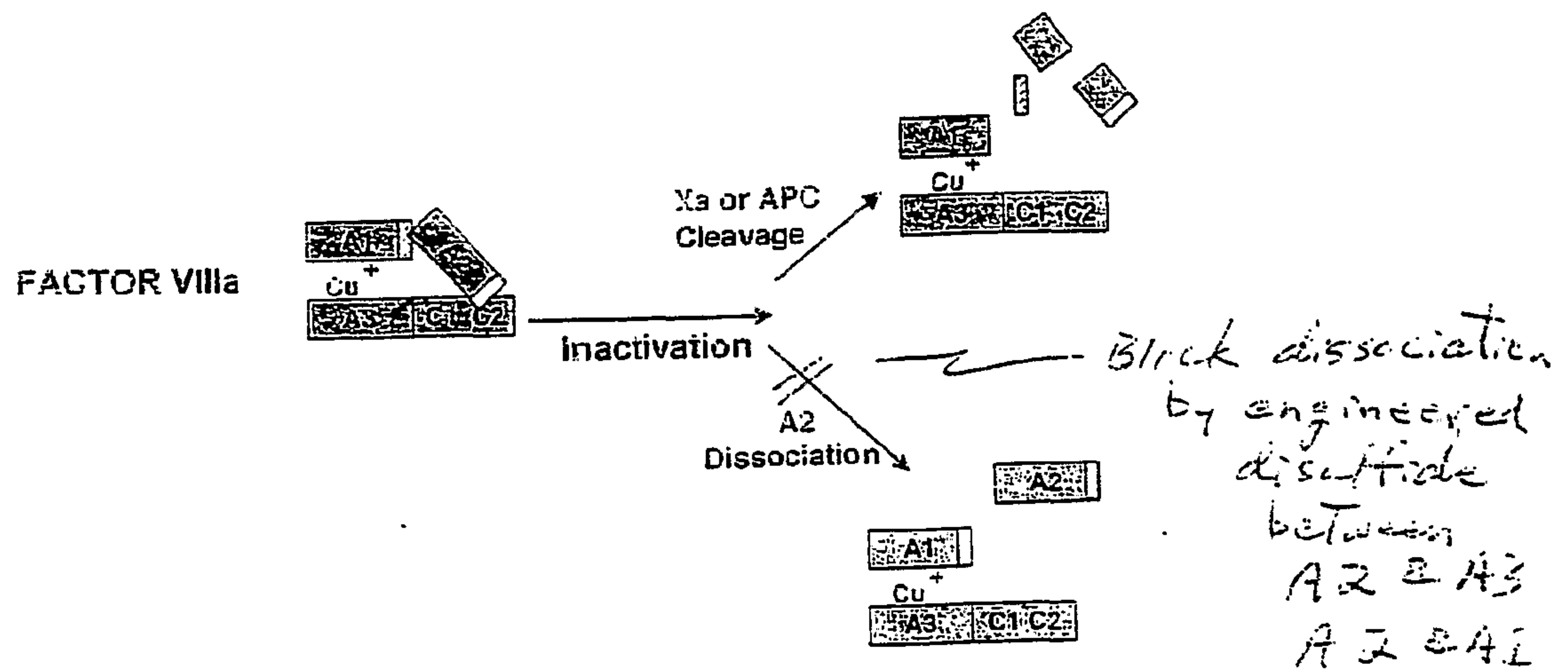


FIG. 3

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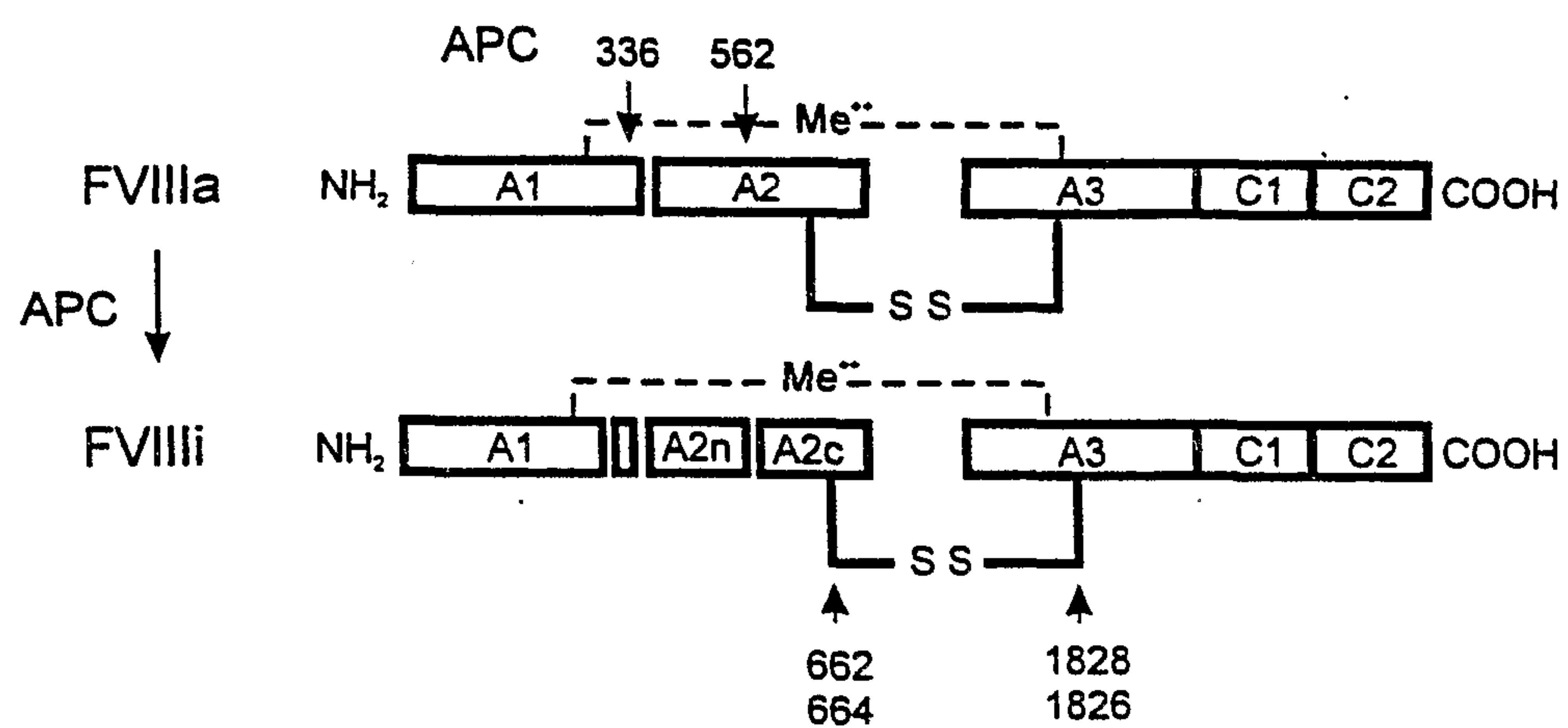


FIG. 4

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Stability of FVIIIa

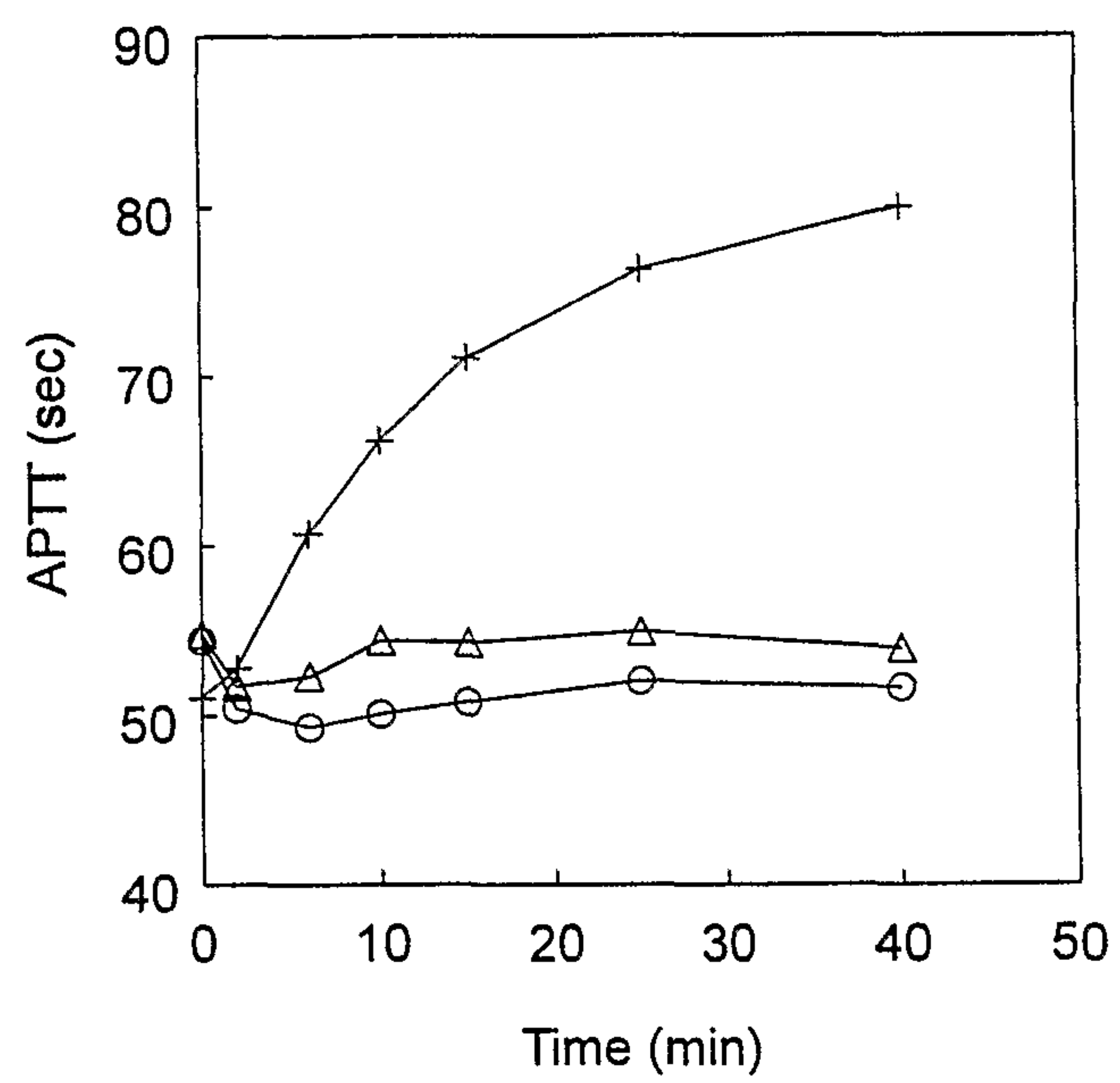


FIG. 5

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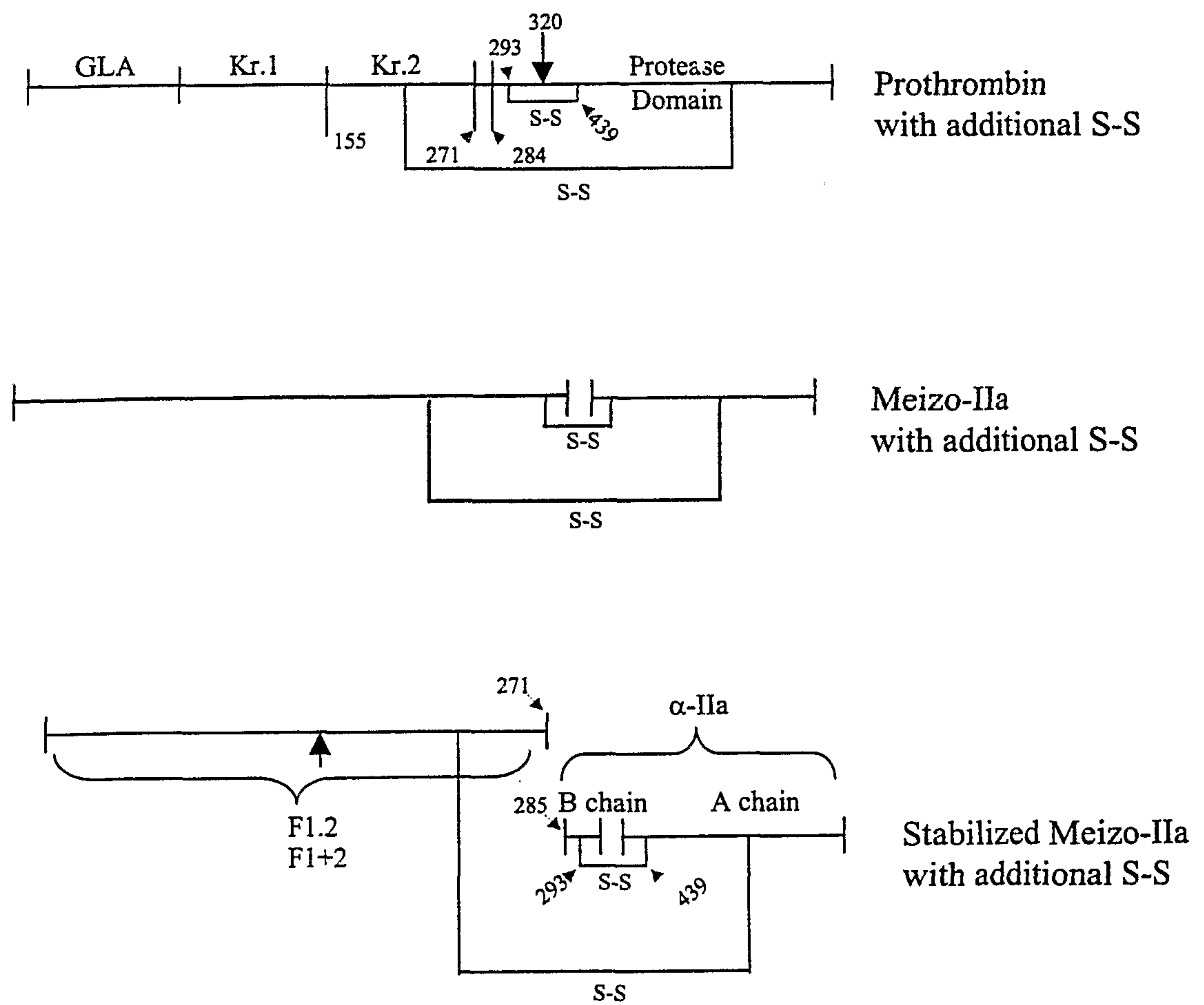


FIG. 6

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Factor VIII

SwissProt Accession # P00451

Refs

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TITLE Expression of active human factor VIII from recombinant DNA clones

JOURNAL Nature 312 (5992), 330-337 (1984)

MEDLINE 85061548

REMARK SEQUENCE FROM N.A.

REFERENCE 3 (residues 1 to 2351)

AUTHORS Toole,J.J., Knopf,J.L., Wozney,J.M., Sultzman,L.A., Buecker,J.L.,
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Amphlett,G.W., Foster,W.B., Coe,M.L., Knutson,G.J., Fass,D.N. and
Hewick,R.M.

TITLE Molecular cloning of a cDNA encoding human antihaemophilic factor

JOURNAL Nature 312 (5992), 342-347 (1984)

Note: Numbering in all of these starts with the first aa of the signal peptide as aa 1. The signal peptide is 19 aa long. Standard numbering of the processed protein starts with the first aa of the processed protein as aa 1 (total aa 2332 instead of 2351) Thus in the swissprot file and the references all residues are numbered 19 higher than we number in the processed protein.

Factor V

SwissProt Accession # P12259

Ref

REFERENCE 2 (residues 1 to 2224)

AUTHORS Jenny,R.J., Pittman,D.D., Toole,J.J., Kriz,R.W., Aldape,R.A.,
Hewick,R.M., Kaufman,R.J. and Mann,K.G.

TITLE Complete cDNA and derived amino acid sequence of human factor V

JOURNAL Proc. Natl. Acad. Sci. U.S.A. 84 (14), 4846-4850 (1987)

Same thing applies to the numbering here as in FVIII. The signal peptide is 28 aa long.

Prothrombin

Swissprot Accession # P00734

Ref

FIG. 7

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REFERENCE 2 (residues 1 to 622)

AUTHORS Degen,S.J., MacGillivray,R.T. and Davie,E.W.

TITLE Characterization of the complementary deoxyribonucleic acid and
gene coding for human prothrombin

JOURNAL Biochemistry 22 (9), 2087-2097 (1983)

Same thing applies to the numbering here as in FVIII. The signal and propeptide together
are 43 aa long and are subtracted from the numbering for the processed polypeptide

Factor XII

Swissprot Acc # P00748

Ref

REFERENCE 3 (residues 1 to 615)

AUTHORS Cool,D.E., Edgell,C.J., Louie,G.V., Zoller,M.J., Brayer,G.D. and
MacGillivray,R.T.TITLE Characterization of human blood coagulation factor XII cDNA.
Prediction of the primary structure of factor XII and the tertiary
structure of beta-factor XIIa

JOURNAL J. Biol. Chem. 260 (25), 13666-13676 (1985)

HGFA

Swissprot Acc# Q04756

Ref

REFERENCE 1 (residues 1 to 655)

AUTHORS Miyazawa,K., Shimomura,T., Kitamura,A., Kondo,J., Morimoto,Y. and
Kitamura,N.TITLE Molecular cloning and sequence analysis of the cDNA for a human
serine protease responsible for activation of hepatocyte growth
factor. Structural similarity of the protease precursor to blood
coagulation factor XII

JOURNAL J. Biol. Chem. 268 (14), 10024-10028 (1993)

PHBP

PIR Acc# JC4795

Ref

REFERENCE 1 (residues 1 to 560)

AUTHORS Choi-Miura,N.H., Tobe,T., Sumiya,J., Nakano,Y., Sano,Y., Mazda,T.
and Tomita,M.

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TITLE Purification and characterization of a novel hyaluronan-binding protein (PHBP) from human plasma: it has three EGF, a kringle and a serine protease domain, similar to hepatocyte growth factor activator
JOURNAL J. Biochem. 119 (6), 1157-1165 (1996)

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NiceProt View of SWISS-PROT: P00451

<http://www.expasy.ch/cgi-bin/niceprot.pl/printable?ac=P00451>

NiceProt View of SWISS-PROT: P00451

General information about the entry

Entry name	FA8_HUMAN
Primary accession number	P00451
Secondary accession numbers	None
Entered in SWISS-PROT in	Release 01, July 1986
Sequence was last modified in	Release 01, July 1986
Annotations were last modified in	Release 40, October 2000

Name and origin of the protein

Protein name	COAGULATION FACTOR VIII [Precursor]
Synonyms	PROCOAGULANT COMPONENT ANTIHEMOPHILIC FACTOR AHF
Gene name	F8 or F8C
From	Homo sapiens (Human) [TaxID: 9606]
Taxonomy	Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.

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FIG. 8

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Comments

- **FUNCTION:** FACTOR VIII, ALONG WITH CALCIUM AND PHOSPHOLIPID, ACTS AS A COFACTOR FOR FACTOR IXA WHEN IT CONVERTS FACTOR X TO THE ACTIVATED FORM, FACTOR XA.
- **SUBCELLULAR LOCATION:** EXTRACELLULAR.
- **DOMAIN:** DOMAIN F5/8 TYPE C 2 IS RESPONSIBLE FOR PHOSPHOLIPID-BINDING AND ESSENTIAL FOR FACTOR VIII ACTIVITY.
- **DISEASE:** HEMOPHILIA A IS A COMMON RECESSIVE X-LINKED COAGULATION DISORDER DUE TO DEFECTS IN F8C. THE FREQUENCY OF HEMOPHILIA A IS 1-2 IN 10,000 MALE BIRTHS IN ALL ETHNIC GROUPS. ABOUT 50% OF PATIENTS HAVE SEVERE HEMOPHILIA A WITH F8C ACTIVITY LESS THAN 1% OF NORMAL; THEY HAVE FREQUENT SPONTANEOUS BLEEDING INTO JOINTS, MUSCLES AND INTERNAL ORGANS. MODERATELY SEVERE HEMOPHILIA A OCCURS IN ABOUT 10% OF PATIENTS; F8C ACTIVITY IS 2-5% OF NORMAL, AND THERE IS BLEEDING AFTER MINOR TRAUMA. MILD HEMOPHILIA A, WHICH OCCURS IN 30-40% OF PATIENTS, IS ASSOCIATED WITH F8C ACTIVITY OF 5-30% AND BLEEDING OCCURS ONLY AFTER SIGNIFICANT TRAUMA OR SURGERY. OF PARTICULAR INTEREST FOR THE UNDERSTANDING OF THE FUNCTION OF F8C IS THE CATEGORY OF CRM (CROSS-REACTING MATERIAL) POSITIVE PATIENTS (APPROXIMATELY 5%) THAT HAVE CONSIDERABLE AMOUNT OF F8C IN THEIR PLASMA (AT LEAST 30% OF NORMAL), BUT THE PROTEIN IS NONFUNCTIONAL; I.E., THE F8C ACTIVITY IS MUCH LESS THAN THE PLASMA PROTEIN LEVEL. CRM-REDUCED IS ANOTHER CATEGORY OF PATIENTS IN WHICH THE F8C ANTIGEN AND ACTIVITY ARE REDUCED TO APPROXIMATELY THE SAME LEVEL. MOST MUTATIONS ARE CRM NEGATIVE, AND PROBABLY AFFECT THE FOLDING AND STABILITY OF THE PROTEIN.
- **PHARMACEUTICAL:** AVAILABLE UNDER THE NAMES KOGENATE (BAYER) AND RECOMBINATE (BAXTER AND AMERICAN HOME PRODUCTS). USED TO TREAT HEMOPHILIA A.
- **SIMILARITY:** CONTAINS 3 F5/8 TYPE A DOMAINS; EACH IS COMPOSED OF 2 PLASTOCYANIN-LIKE REPEATS.
- **SIMILARITY:** CONTAINS 2 F5/8 TYPE C DOMAINS.
- **SIMILARITY:** STRONG, TO COAGULATION FACTOR V.
- **DATABASE:** NAME=HAMSters; NOTE=Factor VIII mutation db; WWW="http://europium.mrc.rpms.ac.uk/".

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Cross-references

EMBL	M14113; AAA52485.1; -. X01179; CAA25619.1; -. K01740; AAA52484.1; -. M88648; AAA52420.1; -. M88628; AAA52420.1; JOINED. M88629; AAA52420.1; JOINED. M88630; AAA52420.1; JOINED. M88631; AAA52420.1; JOINED. M88632; AAA52420.1; JOINED. M88633; AAA52420.1; JOINED. M88634; AAA52420.1; JOINED. M88635; AAA52420.1; JOINED. M88636; AAA52420.1; JOINED. M88638; AAA52420.1; JOINED. M88639; AAA52420.1; JOINED. M88640; AAA52420.1; JOINED. M88641; AAA52420.1; JOINED. M88642; AAA52420.1; JOINED. M88643; AAA52420.1; JOINED. M88644; AAA52420.1; JOINED. M88645; AAA52420.1; JOINED. M88646; AAA52420.1; JOINED. M88647; AAA52420.1; JOINED. U80228; AAB61261.1; -.
PIR	A00525; EZHU. A23584; A23584.
PDB	1CFG; 02-NOV-95. 1FAC; 11-JAN-97.
GlycoSuiteDB	P00451; -.
MIM	134430 . 134500 . 134510 . 227310 . 306700 .
GeneCards	F8.
InterPro	IPR001117; Cu-oxidase. IPR000421; FA58_C.
Pfam	PF00394; Cu-oxidase; 3. PF00754; F5_F8_type_C; 2.
SMART	SM00231; FA58C; 2.
PROSITE	PS00079; MULTICOPPER_OXIDASE1; 3. PS01285; FA58C_1; 2. PS01286; FA58C_2; 2.
Implicit links to	ProDom; BLOCKS; DOMO; ProtoMap; PRESAGE; DIP; SWISS-2DPAGE.

Keywords

Blood coagulation; Repeat; Plasma; Acute phase; Calcium; Hemophilia; Signal; Glycoprotein; Sulfation; Disease mutation; Polymorphism; Pharmaceutical; 3D-structure.

Features

Key	From	To	Length	Description
SIGNAL	1	19	19	
CHAIN	20	2351	2332	COAGULATION FACTOR VIII.
CHAIN	20	1332	1313	FACTOR VIIIA HEAVY CHAIN (200 KDA).
CHAIN	20	759	740	FACTOR VIIIA HEAVY CHAIN (92 KDA).
CHAIN	760	1332	573	B.
CHAIN	1668	2351	684	FACTOR VIIIA LIGHT CHAIN (80 KDA).
DOMAIN	20	348	329	F5/8 TYPE A 1.
DOMAIN	20	198	179	PLASTOCYANIN-LIKE 1.
DOMAIN	206	348	143	PLASTOCYANIN-LIKE 2.
DOMAIN	399	730	332	F5/8 TYPE A 2.
DOMAIN	399	573	175	PLASTOCYANIN-LIKE 3.
DOMAIN	583	730	148	PLASTOCYANIN-LIKE 4.
DOMAIN	760	1667	908	B.
DOMAIN	1713	2040	328	F5/8 TYPE A 3.
DOMAIN	1713	1877	165	PLASTOCYANIN-LIKE 5.
DOMAIN	1887	2040	154	PLASTOCYANIN-LIKE 6.
DOMAIN	2040	2188	149	F5/8 TYPE C 1.
DOMAIN	2193	2345	153	F5/8 TYPE C 2.
SITE	1332	1333	2	CLEAVAGE (ACTIVATION).
SITE	1667	1668	2	CLEAVAGE (ACTIVATION).
SITE	391	392	2	CLEAVAGE (BY THROMBIN).
SITE	759	760	2	CLEAVAGE (BY THROMBIN).
SITE	1708	1709	2	CLEAVAGE (BY THROMBIN).
MOD_RES	365	365		SULFATION.
MOD_RES	737	737		SULFATION (PROBABLE).
MOD_RES	738	738		SULFATION (PROBABLE).
MOD_RES	742	742		SULFATION (PROBABLE).
MOD_RES	1683	1683		SULFATION.
MOD_RES	1699	1699		SULFATION.
DISULFID	172	198		PROBABLE.
DISULFID	547	573		PROBABLE.
DISULFID	1851	1877		PROBABLE.
DISULFID	2040	2188		BY SIMILARITY.
DISULFID	2193	2345		BY SIMILARITY.
CARBOHYD	60	60		N-LINKED (GLCNAC...) (POTENTIAL).
CARBOHYD	258	258		N-LINKED (GLCNAC...) (POTENTIAL).
CARBOHYD	601	601		N-LINKED (GLCNAC...) (POTENTIAL).
CARBOHYD	776	776		N-LINKED (GLCNAC...) (POTENTIAL).
CARBOHYD	803	803		N-LINKED (GLCNAC...) (POTENTIAL).
CARBOHYD	847	847		N-LINKED (GLCNAC...) (POTENTIAL).
CARBOHYD	919	919		N-LINKED (GLCNAC...) (POTENTIAL).
CARBOHYD	962	962		N-LINKED (GLCNAC...) (POTENTIAL).
CARBOHYD	982	982		N-LINKED (GLCNAC...) (POTENTIAL).
CARBOHYD	1020	1020		N-LINKED (GLCNAC...) (POTENTIAL).
CARBOHYD	1024	1024		N-LINKED (GLCNAC...) (POTENTIAL).
CARBOHYD	1074	1074		N-LINKED (GLCNAC...) (POTENTIAL).
CARBOHYD	1085	1085		N-LINKED (GLCNAC...) (POTENTIAL).
CARBOHYD	1204	1204		N-LINKED (GLCNAC...) (POTENTIAL).
CARBOHYD	1274	1274		N-LINKED (GLCNAC...) (POTENTIAL).
CARBOHYD	1278	1278		N-LINKED (GLCNAC...) (POTENTIAL).
CARBOHYD	1301	1301		N-LINKED (GLCNAC...) (POTENTIAL).
CARBOHYD	1319	1319		N-LINKED (GLCNAC...) (POTENTIAL).
CARBOHYD	1431	1431		N-LINKED (GLCNAC...) (POTENTIAL).
CARBOHYD	1461	1461		N-LINKED (GLCNAC...) (POTENTIAL).
CARBOHYD	1829	1829		N-LINKED (GLCNAC...) (POTENTIAL).
CARBOHYD	2137	2137		N-LINKED (GLCNAC...) (POTENTIAL).

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<http://www.expasy.ch/cgi-bin/niceprot.pl:pnntable?ac=P00451>

VARIANT	26	26	L -> R (SEVERE HEMOPHILIA) . /FTid=VAR_001045.
VARIANT	30	30	E -> V (MILD HEMOPHILIA) . /FTid=VAR_001046.
VARIANT	41	41	G -> C (SEVERE/MODERATE HEMOPHILIA) . /FTid=VAR_001047.
VARIANT	48	48	R -> C (SEVERE HEMOPHILIA) . /FTid=VAR_001048.
VARIANT	75	75	D -> V . /FTid=VAR_001049.
VARIANT	89	89	G -> D (SEVERE HEMOPHILIA) . /FTid=VAR_001050.
VARIANT	89	89	G -> V (MILD HEMOPHILIA) . /FTid=VAR_001051.
VARIANT	99	99	V -> D (SEVERE HEMOPHILIA) . /FTid=VAR_001052.
VARIANT	104	104	V -> D (MILD HEMOPHILIA) . /FTid=VAR_001053.
VARIANT	108	108	K -> T (MILD HEMOPHILIA) . /FTid=VAR_001054.
VARIANT	110	110	M -> V (MODERATE HEMOPHILIA) . /FTid=VAR_001055.
VARIANT	117	117	L -> R (SEVERE HEMOPHILIA) . /FTid=VAR_001056.
VARIANT	129	129	E -> V (SEVERE HEMOPHILIA) . /FTid=VAR_001057.
VARIANT	130	130	G -> R (SEVERE HEMOPHILIA) . /FTid=VAR_001058.
VARIANT	132	132	E -> D (SEVERE HEMOPHILIA) . /FTid=VAR_001059.
VARIANT	133	133	Y -> C (MILD HEMOPHILIA) . /FTid=VAR_001060.
VARIANT	135	135	D -> G (SEVERE HEMOPHILIA) . /FTid=VAR_001061.
VARIANT	137	137	T -> I (MODERATE HEMOPHILIA) . /FTid=VAR_001062.
VARIANT	164	164	G -> V (MILD HEMOPHILIA) . /FTid=VAR_001063.
VARIANT	165	165	P -> S (SEVERE HEMOPHILIA) . /FTid=VAR_001064.
VARIANT	181	181	V -> M (MODERATE HEMOPHILIA) . /FTid=VAR_001065.
VARIANT	185	185	K -> T (MILD HEMOPHILIA) . /FTid=VAR_001066.
VARIANT	189	189	S -> L (MODERATE HEMOPHILIA) . /FTid=VAR_001067.
VARIANT	202	202	S -> R (MILD HEMOPHILIA) . /FTid=VAR_008123.
VARIANT	222	222	D -> V (MODERATE HEMOPHILIA) . /FTid=VAR_001068.
VARIANT	224	224	G -> W (MODERATE HEMOPHILIA) . /FTid=VAR_001069.
VARIANT	253	253	V -> F (SEVERE HEMOPHILIA) . /FTid=VAR_001070.
VARIANT	266	266	G -> Q (SEVERE HEMOPHILIA) . /FTid=VAR_001071.
VARIANT	278	278	G -> R (SEVERE HEMOPHILIA) . /FTid=VAR_001072.
VARIANT	285	285	V -> G (MILD HEMOPHILIA) . /FTid=VAR_001073.
VARIANT	291	291	E -> G (MODERATE/MILD HEMOPHILIA) . /FTid=VAR_001074.
VARIANT	294	294	T -> I (MODERATE HEMOPHILIA) . /FTid=VAR_001075.
VARIANT	299	299	N -> I (MILD HEMOPHILIA) . /FTid=VAR_001076.
VARIANT	301	301	R -> H (SEVERE HEMOPHILIA) . /FTid=VAR_001077.
VARIANT	301	301	R -> L (SEVERE HEMOPHILIA) . /FTid=VAR_001078.
VARIANT	308	308	S -> L (MODERATE HEMOPHILIA) . /FTid=VAR_001079.
VARIANT	312	312	F -> S (MODERATE HEMOPHILIA) . /FTid=VAR_001080.
VARIANT	314	314	T -> A (MILD HEMOPHILIA) . /FTid=VAR_001081.
VARIANT	314	314	T -> I (MODERATE HEMOPHILIA) . /FTid=VAR_001082.
VARIANT	327	327	L -> P (SEVERE HEMOPHILIA) . /FTid=VAR_001083.

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VARIANT	331	331	I -> V (MILD HEMOPHILIA). /FTid=VAR_001084.
VARIANT	345	345	V -> L (SEVERE HEMOPHILIA). /FTid=VAR_001085.
VARIANT	348	348	C -> R (SEVERE HEMOPHILIA). /FTid=VAR_001086.
VARIANT	348	348	C -> S (MODERATE HEMOPHILIA). /FTid=VAR_001087.
VARIANT	348	348	C -> Y (SEVERE HEMOPHILIA). /FTid=VAR_001088.
VARIANT	391	391	R -> C (OKAYAMA; MODERATE HEMOPHILIA; ABOLISHES THE NORMAL CLEAVAGE BY THROMBIN). /FTid=VAR_001089.
VARIANT	391	391	R -> H (KUMAMOTO; MODERATE/MILD HEMOPHILIA; ABOLISHES THE NORMAL CLEAVAGE BY THROMBIN). /FTid=VAR_001090.
VARIANT	391	391	R -> P (SEVERE HEMOPHILIA; ABOLISHES THE NORMAL CLEAVAGE BY THROMBIN). /FTid=VAR_001091.
VARIANT	392	392	S -> L (MILD HEMOPHILIA; ABOLISHES NORMAL CLEAVAGE BY THROMBIN). /FTid=VAR_001092.
VARIANT	392	392	S -> P (MILD HEMOPHILIA). /FTid=VAR_001093.
VARIANT	405	405	I -> S (SEVERE HEMOPHILIA). /FTid=VAR_001094.
VARIANT	409	409	E -> G (SEVERE/MODERATE HEMOPHILIA). /FTid=VAR_001095.
VARIANT	431	431	L -> F (MODERATE/MILD HEMOPHILIA). /FTid=VAR_001096.
VARIANT	439	439	G -> V (SEVERE HEMOPHILIA). /FTid=VAR_001097.
VARIANT	444	444	K -> R (SEVERE HEMOPHILIA). /FTid=VAR_001098.
VARIANT	450	450	Y -> N (MODERATE HEMOPHILIA). /FTid=VAR_001099.
VARIANT	474	474	G -> R (SEVERE HEMOPHILIA). /FTid=VAR_001100.
VARIANT	488	488	A -> G (MODERATE HEMOPHILIA). /FTid=VAR_001101.
VARIANT	492	492	Y -> H (MILD HEMOPHILIA). /FTid=VAR_001102.
VARIANT	492	492	Y -> C (MODERATE HEMOPHILIA). /FTid=VAR_001103.
VARIANT	494	494	I -> T (MILD HEMOPHILIA). /FTid=VAR_001104.
VARIANT	498	498	G -> R (SEVERE/MODERATE HEMOPHILIA). /FTid=VAR_001105.
VARIANT	544	544	D -> N (MODERATE HEMOPHILIA). /FTid=VAR_001106.
VARIANT	546	546	R -> W (MILD HEMOPHILIA). /FTid=VAR_001107.
VARIANT	550	550	R -> C (MODERATE/MILD HEMOPHILIA). /FTid=VAR_001108.
VARIANT	550	550	R -> G (MILD HEMOPHILIA). /FTid=VAR_001109.
VARIANT	550	550	R -> H (MILD HEMOPHILIA). /FTid=VAR_001110.
VARIANT	554	554	S -> G (MILD HEMOPHILIA). /FTid=VAR_001111.
VARIANT	556	556	V -> D (MODERATE HEMOPHILIA). /FTid=VAR_001112.
VARIANT	561	561	D -> Y (IN SEVERE HEMOPHILIA). /FTid=VAR_008967.
VARIANT	577	577	S -> F (MILD HEMOPHILIA). /FTid=VAR_001113.
VARIANT	584	584	Q -> K (MODERATE/MILD HEMOPHILIA). /FTid=VAR_001114.
VARIANT	585	585	I -> T (SEVERE/MODERATE HEMOPHILIA). /FTid=VAR_001115.
VARIANT	596	596	S -> P (SEVERE HEMOPHILIA). /FTid=VAR_001116.
VARIANT	603	603	S -> I (HEMOPHILIA). /FTid=VAR_001117.
VARIANT	604	604	W -> C (SEVERE HEMOPHILIA). /FTid=VAR_001118.
VARIANT	605	605	Y -> S (SEVERE HEMOPHILIA). /FTid=VAR_001119.
VARIANT	612	612	R -> C (MILD/MODERATE HEMOPHILIA). /FTid=VAR_001120.

VARIANT	631	631	N -> K (SEVERE HEMOPHILIA). /FTid=VAR_001121.
VARIANT	631	631	N -> S (HEMOPHILIA). /FTid=VAR_001122.
VARIANT	644	644	L -> V (MILD HEMOPHILIA). /FTid=VAR_001123.
VARIANT	653	653	V -> A (MILD HEMOPHILIA). /FTid=VAR_001124.
VARIANT	653	653	V -> M (SEVERE HEMOPHILIA). /FTid=VAR_001125.
VARIANT	663	663	A -> V (MILD HEMOPHILIA). /FTid=VAR_001126.
VARIANT	671	671	MISSING (SEVERE HEMOPHILIA). /FTid=VAR_001127.
VARIANT	677	677	F -> L (MODERATE HEMOPHILIA). /FTid=VAR_001128.
VARIANT	699	699	M -> V (SEVERE HEMOPHILIA). /FTid=VAR_001129.
VARIANT	717	717	R -> W (MILD HEMOPHILIA). /FTid=VAR_001130.
VARIANT	720	720	G -> D (SEVERE/MODERATE HEMOPHILIA). /FTid=VAR_001131.
VARIANT	723	723	A -> T (MILD/MODERATE HEMOPHILIA). /FTid=VAR_001132.
VARIANT	727	727	V -> F (SEVERE HEMOPHILIA). /FTid=VAR_001133.
VARIANT	739	739	E -> K (MILD HEMOPHILIA). /FTid=VAR_001134.
VARIANT	1057	1057	E -> K (MODERATE HEMOPHILIA). /FTid=VAR_001135.
VARIANT	1260	1260	D -> E. /FTid=VAR_001136.
VARIANT	1481	1481	L -> P. /FTid=VAR_001137.
VARIANT	1699	1699	Y -> C (SEVERE HEMOPHILIA). /FTid=VAR_001138.
VARIANT	1699	1699	Y -> F (MILD/MODERATE HEMOPHILIA). /FTid=VAR_001139.
VARIANT	1708	1708	R -> C (EAST HARTFORD; SEVERE/MODERATE HEMOPHILIA; ABOLISHES THROMBIN CLEAVAGE AT THE LIGHT CHAIN). /FTid=VAR_001140.
VARIANT	1708	1708	R -> H (MILD HEMOPHILIA; ABOLISHES THROMBIN CLEAVAGE AT THE LIGHT CHAIN). /FTid=VAR_001141.
VARIANT	1715	1715	R -> G (MILD HEMOPHILIA). /FTid=VAR_001142.
VARIANT	1723	1723	E -> K (SEVERE HEMOPHILIA). /FTid=VAR_001143.
VARIANT	1728	1728	Y -> C (MODERATE HEMOPHILIA). /FTid=VAR_001144.
VARIANT	1769	1769	G -> R (MILD HEMOPHILIA). /FTid=VAR_001145.
VARIANT	1775	1775	L -> V (MODERATE HEMOPHILIA). /FTid=VAR_001146.
VARIANT	1775	1775	L -> F (MILD HEMOPHILIA). /FTid=VAR_001147.
VARIANT	1779	1779	G -> E (SEVERE HEMOPHILIA). /FTid=VAR_001148.
VARIANT	1791	1791	M -> T (SEVERE HEMOPHILIA). /FTid=VAR_001149.
VARIANT	1800	1800	R -> H (MODERATE HEMOPHILIA). /FTid=VAR_001150.
VARIANT	1800	1800	R -> C (MILD HEMOPHILIA). /FTid=VAR_001151.
VARIANT	1800	1800	R -> G (MILD HEMOPHILIA). /FTid=VAR_001152.
VARIANT	1803	1803	S -> Y (SEVERE HEMOPHILIA). /FTid=VAR_001153.
VARIANT	1808	1808	L -> F (MILD HEMOPHILIA). /FTid=VAR_001154.
VARIANT	1842	1842	M -> I (MODERATE HEMOPHILIA). /FTid=VAR_001155.
VARIANT	1844	1844	P -> S (MILD HEMOPHILIA). /FTid=VAR_001156.
VARIANT	1845	1845	T -> P (MILD HEMOPHILIA). /FTid=VAR_001157.
VARIANT	1853	1853	A -> T (SEVERE HEMOPHILIA). /FTid=VAR_001158.
VARIANT	1853	1853	A -> V (MILD HEMOPHILIA). /FTid=VAR_001159.

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VARIANT	1865	1865	D -> N (SEVERE HEMOPHILIA). /FTid=VAR_001160.
VARIANT	1865	1865	D -> Y (SEVERE HEMOPHILIA). /FTid=VAR_001161.
VARIANT	1867	1867	H -> R (MODERATE HEMOPHILIA). /FTid=VAR_001162.
VARIANT	1869	1869	G -> V (SEVERE HEMOPHILIA). /FTid=VAR_001163.
VARIANT	1873	1873	P -> R (SEVERE HEMOPHILIA). /FTid=VAR_001164.
VARIANT	1888	1888	R -> I (SEVERE HEMOPHILIA). /FTid=VAR_001165.
VARIANT	1894	1894	E -> G (MODERATE HEMOPHILIA). /FTid=VAR_001166.
VARIANT	1904	1904	E -> K (SEVERE HEMOPHILIA). /FTid=VAR_001167.
VARIANT	1941	1941	N -> D (SEVERE/MODERATE HEMOPHILIA). /FTid=VAR_001168.
VARIANT	1941	1941	N -> S (SEVERE/MODERATE HEMOPHILIA). /FTid=VAR_001169.
VARIANT	1960	1960	R -> Q (MILD/MODERATE HEMOPHILIA). /FTid=VAR_001170.
VARIANT	1960	1960	R -> L (MODERATE HEMOPHILIA). /FTid=VAR_001171.
VARIANT	1967	1967	G -> D (MODERATE HEMOPHILIA). /FTid=VAR_001172.
VARIANT	1979	1979	G -> V (MODERATE HEMOPHILIA). /FTid=VAR_001173.
VARIANT	1980	1980	H -> Y (MILD HEMOPHILIA). /FTid=VAR_001174.
VARIANT	2016	2016	R -> W (MODERATE/SEVERE HEMOPHILIA). /FTid=VAR_001175.
VARIANT	2038	2038	N -> S (MODERATE HEMOPHILIA). /FTid=VAR_001176.
VARIANT	2065	2065	W -> R (MODERATE HEMOPHILIA). /FTid=VAR_001177.
VARIANT	2088	2088	S -> F (SEVERE HEMOPHILIA). /FTid=VAR_001178.
VARIANT	2093	2093	D -> G (MILD HEMOPHILIA). /FTid=VAR_001179.
VARIANT	2105	2105	T -> N (MODERATE HEMOPHILIA). /FTid=VAR_001180.
VARIANT	2107	2107	G -> S (SEVERE HEMOPHILIA). /FTid=VAR_001181.
VARIANT	2120	2120	F -> L (MILD HEMOPHILIA). /FTid=VAR_001182.
VARIANT	2124	2124	Y -> C (MILD HEMOPHILIA). /FTid=VAR_001183.
VARIANT	2135	2135	R -> P (SEVERE HEMOPHILIA). /FTid=VAR_001184.
VARIANT	2138	2138	S -> Y (MILD/MODERATE HEMOPHILIA). /FTid=VAR_001185.
VARIANT	2148	2148	N -> S (MODERATE HEMOPHILIA). /FTid=VAR_001186.
VARIANT	2169	2169	R -> H (SEVERE/MILD HEMOPHILIA). /FTid=VAR_001187.
VARIANT	2172	2172	P -> Q (MODERATE HEMOPHILIA). /FTid=VAR_001188.
VARIANT	2173	2173	T -> I (MILD HEMOPHILIA). /FTid=VAR_001189.
VARIANT	2178	2178	R -> C (MILD HEMOPHILIA). /FTid=VAR_001190.
VARIANT	2178	2178	R -> H (MILD HEMOPHILIA). /FTid=VAR_001191.
VARIANT	2178	2178	R -> L (MILD HEMOPHILIA). /FTid=VAR_001192.
VARIANT	2182	2182	R -> C (MODERATE HEMOPHILIA). /FTid=VAR_001193.
VARIANT	2182	2182	R -> H (MODERATE/SEVERE HEMOPHILIA). /FTid=VAR_001194.
VARIANT	2183	2183	M -> V (MILD HEMOPHILIA). /FTid=VAR_001195.
VARIANT	2185	2185	L -> S (SEVERE HEMOPHILIA). /FTid=VAR_001196.
VARIANT	2204	2204	I -> T (MILD HEMOPHILIA). /FTid=VAR_001197.
VARIANT	2209	2209	I -> N (MODERATE HEMOPHILIA). /FTid=VAR_001198.
VARIANT	2211	2211	A -> P (MODERATE HEMOPHILIA). /FTid=VAR_001199.

VARIANT	2223	2223	MISSING (SEVERE/MODERATE HEMOPHILIA). /FTid=VAR_001200.
VARIANT	2228	2228	R -> G (SEVERE HEMOPHILIA). /FTid=VAR_001201.
VARIANT	2228	2228	R -> L (MODERATE HEMOPHILIA). /FTid=VAR_001202.
VARIANT	2228	2228	R -> Q (SEVERE/MODERATE HEMOPHILIA). /FTid=VAR_001203.
VARIANT	2242	2242	V -> M. /FTid=VAR_001204.
VARIANT	2248	2248	W -> C (MODERATE HEMOPHILIA). /FTid=VAR_001205.
VARIANT	2265	2265	Q -> R (MODERATE HEMOPHILIA). /FTid=VAR_001206.
VARIANT	2319	2319	P -> L (MILD/SEVERE HEMOPHILIA). /FTid=VAR_001207.
VARIANT	2319	2319	P -> S (MILD HEMOPHILIA). /FTid=VAR_001208.
VARIANT	2323	2323	R -> C (SEVERE HEMOPHILIA; MAY CAUSE REDUCED PHOSPHOLIPID BINDING). /FTid=VAR_001209.
VARIANT	2323	2323	R -> H (MILD HEMOPHILIA; MAY CAUSE REDUCED PHOSPHOLIPID BINDING). /FTid=VAR_001210.
VARIANT	2326	2326	R -> L (SEVERE/MODERATE HEMOPHILIA; MAY CAUSE REDUCED PHOSPHOLIPID BINDING). /FTid=VAR_001211.
VARIANT	2326	2326	R -> Q (MILD/MODERATE HEMOPHILIA; MAY CAUSE REDUCED PHOSPHOLIPID BINDING). /FTid=VAR_001212.
VARIANT	2344	2344	G -> C (MODERATE HEMOPHILIA). /FTid=VAR_008968.
CONFLICT	768	768	P -> R (IN REF. 2).
CONFLICT	1922	1922	C -> S (IN REF. 4).

Sequence information

Length: 2351 AA [This is the length of the unprocessed precursor]
Molecular weight: 267007 Da [This is the MW of the unprocessed precursor]
CRC64: 75FB6A2955C74CB0 [This is a checksum on the sequence]

10	20	30	40	50	60
MQIELSTCFF	LCLLRFCFSA	TRRYYLGAVE	LSWDYMQSDL	GELPVDARFP	PRVPKSFPPN
70	80	90	100	110	120
TSVVYKKTLE	VEFTDHLFNI	AKPRPPWMGL	LGPTIQAEVY	DTVVITLKNM	ASHPVSLHAV
130	140	150	160	170	180
GVSYWKASEG	AEYDDQTSQR	EKEDDKVFPG	GSHTYVWQVL	KENGPMASDP	LCLTYSYLSH
190	200	210	220	230	240
VDLVKDLNSG	LIGALLVCRE	GSLAKEKTQT	LHKFILLFAV	FDEGKSWHSE	TKNSLMQDRD
250	260	270	280	290	300
AASARAWPKM	HTVNGYVNRS	LPGLIGCHRK	SVYWHVIGMG	TTPEVHSIFL	EGHTFLVRNH
310	320	330	340	350	360
RQASLEISPI	TFLTAQTLLM	DLGQFLLFCH	ISSHQHDGME	AYVKVDSCPE	EPQLRMKNNE
370	380	390	400	410	420
EAEDYDDDLT	DSEMDVVRFD	DDNSPSFIQI	RSVAKKHPKT	WVHYIAAEEE	DWDYAPLVLA
430	440	450	460	470	480
PDDRSYKSQY	LNNGPQRIGR	KYKKVRFMAY	TDETFKTREA	IQHESGILGP	LLYGEVGDTL
490	500	510	520	530	540
LIIFKNQASR	PYNIYPHGIT	DVRPLYSRRL	PKGKHLKDF	PILPGEIFKY	KWTVTVEDGP
550	560	570	580	590	600
TKSDPRCLTR	YYSSFVNMER	DLASGLIGPL	LICYKESVDQ	RGNQIMSDKR	NVILFSVFDE

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610      620      630      640      650      660
|      |      |      |      |      |
NRSWYLTENI QRFLPNPAGV QLEDPEFQAS NIMHSINGYV FDSLQLSVCL HEVAYWYILS

670      680      690      700      710      720
|      |      |      |      |      |
IGAQTDFLSV FFSGYTFKHK MVYEDTLTLF PFSGETVFMS MENPGLWILG CHNSDFRNRG

730      740      750      760      770      780
|      |      |      |      |      |
MTALLKVSSC DKNTGDYYED SYEDISAYLL SKNNAIEPRS FSQNSRHPST RQKQFNATTI

790      800      810      820      830      840
|      |      |      |      |      |
PENDIEKTFD WFAHRTMPMK IQNVSSSDLL MLLRQSPTPH GLSLSDLQFA KYETFSDDPS

850      860      870      880      890      900
|      |      |      |      |      |
PGAIDSNNSL SEMTHFRPQL HHSGDMVFTP ESGLQLRLNE KLGTTAATEL KKLDKVSST

910      920      930      940      950      960
|      |      |      |      |      |
SNNLISTIPS DNLAAGTDNT SSLGPPSMPV HYDSQLDTTL FGKKSSPLTE SGGPLSLSEE

970      980      990      1000      1010      1020
|      |      |      |      |      |
NNDSKLLESG LMNSQESSWG KNVSSTESGR LFKGKRAHGP ALLTKDNALF KVSISLLKTN

1030      1040      1050      1060      1070      1080
|      |      |      |      |      |
KTSNNSATNR KTHIDGPSLL IENSPSVWQN ILESDETFKK VTPLIHDRML MDKNATALRL

1090      1100      1110      1120      1130      1140
|      |      |      |      |      |
NHMSNKTSS KNMEMVQQKK EGPIPPDAQN PDMSFFKMLF LPESARWIQR THGKNSLNSG

1150      1160      1170      1180      1190      1200
|      |      |      |      |      |
QGSPKQLVS LGPEKSVGQ NFLSEKNKV VVGKGEFTKDV GLKEMVFPSS RNLFLTNDLN

1210      1220      1230      1240      1250      1260
|      |      |      |      |      |
LHENNTHNQE KKIQEEIEKK ETLIQENVVL PQIHTVTGTK NFMKNLFLLS TRQNVEGSYD

1270      1280      1290      1300      1310      1320
|      |      |      |      |      |
GAYAPVLQDF RSLNDSTNRT KKHTAHFSKK GEEENLEGLG NQTKQIVEKY ACTTRISPNT

1330      1340      1350      1360      1370      1380
|      |      |      |      |      |
SQQNFTQRS KRALKQFRLP LEETELEKRI IVDDTSTQWS KNMKHLTPST LTQIDYNEKE

1390      1400      1410      1420      1430      1440
|      |      |      |      |      |
KGAITQSPLS DCLTRSHSIP QANRSPLPIA KVSSFPSIRP IYLTRVLFQD NSSHLPAASY

1450      1460      1470      1480      1490      1500
|      |      |      |      |      |
RKKDSGVQES SHFLQGAKKN NLSLAILTLE MTGDQREVGS LGTSATNSVT YKKVENTVLP

1510      1520      1530      1540      1550      1560
|      |      |      |      |      |
KPDLPKTSBK VELLPKVHIY QKDLFPTETS NGSPGHLDLV EGSLLOGTEG AIKWNEANRP

1570      1580      1590      1600      1610      1620
|      |      |      |      |      |
GKVPFLRVAT ESSAKTPSKL LDPLAWDNHY GTQIPKEEWK SQEKSPEKTA FKKKDTILSL

1630      1640      1650      1660      1670      1680
|      |      |      |      |      |
NACESNHAIA AINEGQNKPE IEVTWAKQGR TERLCSQNPV VLKRHQREIT RTTLQSDQEE

1690      1700      1710      1720      1730      1740
|      |      |      |      |      |
IDYDDTISVE MKKEDFDIYD EDENQSPRSF QKKTRHYFIA AVERLWDYGM SSSPHVLRNR

1750      1760      1770      1780      1790      1800
|      |      |      |      |      |
AQSGSVPQFK KVVFEFTDG SFTQPLYRGE LNEHLGLLGP YIRAEVEDNI MVTFRNQASR

1810      1820      1830      1840      1850      1860
|      |      |      |      |      |
PYSFYSSLIS YEEDQRQGAE PRKNEVKPNE TKTYFWKVQH HMAPTKDEFD CKAWAYFSDV
```

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1870	1880	1890	1900	1910	1920
DLEKDVHSGL	IGPLLVCHTN	TLNPAHGRQV	TVQEFALFFT	IFDETKSWYF	TENMERN CRA
1930	1940	1950	1960	1970	1980
PCNIQMEDPT	FKENYRFHAI	NGYIMDTLPG	LVMAQDQRIR	WYLLSMGSNE	NIHSIHFS GH
1990	2000	2010	2020	2030	2040
VFTVRKKEEY	KMALYNLYPG	VFETVEMLPS	KAGIWRVECL	IGEHLHAGMS	TLFLVYSN KC
2050	2060	2070	2080	2090	2100
QTPLGMASGH	IRDFQITASG	QYGQWAPKLA	RLHYSGSINA	WSTKEPFSWI	KVDLLAPM II
2110	2120	2130	2140	2150	2160
HGIKTQGARQ	KFSSLYISQF	IIMYSLDGKK	WQTYRGNSTG	TLMVFFGNVD	SSGIKHNIF N
2170	2180	2190	2200	2210	2220
PPIIARYIRL	HPTHYSIRST	LRMELMGCDL	NSCSMPLGME	SKAISDAQIT	ASSYFTNMFA
2230	2240	2250	2260	2270	2280
TWSPSKARLH	LQGRSNAWRP	QVNNPKEWLQ	VDFQKTMKVT	GVTTQGVKSL	LTSMYVKEFL
2290	2300	2310	2320	2330	2340
ISSSQDGHQW	TLFFQNGKVK	VFOGNQDSFT	PVVNSLDPPL	LTRYLRIHPQ	SWVHQIALRM
2350					
EVLGCEAQDL	Y				

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[\[Sequence\]](#) [\[Tools\]](#)

General information about the entry

Entry name	FA5_HUMAN
Primary accession number	P12259
Secondary accession number	Q14285
Entered in SWISS-PROT in	Release 12, October 1989
Sequence was last modified in	Release 28, February 1994
Annotations were last modified in	Release 40, October 2000

Name and origin of the protein

Protein name	COAGULATION FACTOR V [Precursor]
Synonym	ACTIVATED PROTEIN C COFACTOR
Gene name	F5
From	Homo sapiens (Human) [TaxID: 9606]
Taxonomy	Eukaryota ; Metazoa ; Chordata ; Craniata ; Vertebrata ; Euteleostomi ; Mammalia ; Eutheria ; Primates ; Catarrhini ; Hominidae ; Homo .

References

FIG. 9

- [1] SEQUENCE FROM NUCLEIC ACID.
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Comments

- **FUNCTION:** COAGULATION FACTOR V IS A COFACTOR THAT PARTICIPATES WITH FACTOR XA TO ACTIVATE PROTHROMBIN TO THROMBIN.
- **SUBUNIT:** FACTOR VA IS COMPOSED OF AN HEAVY CHAIN AND OF A LIGHT CHAIN NONCOVALENTLY BOUND. THE INTERACTION BETWEEN THE TWO CHAINS IS CALCIUM-DEPENDENT.
- **DOMAIN:** DOMAIN B CONTAINS 35 X 9 AA TANDEM REPEATS, AND 2 X 17 AA REPEATS.
- **PTM:** THROMBIN ACTIVATES FACTOR V PROTEOLYTICALLY TO THE ACTIVE COFACTOR, FACTOR V(A) (FORMATION OF A HEAVY CHAIN AT THE N-TERMINUS AND A LIGHT CHAIN AT THE C-TERMINUS).
- **DISEASE:** OWREN PARAHEMOPHILIA, AN HEMORRHAGIC DIASTESIS, IS DUE TO A DEFICIENCY OF FACTOR V. OTHER DEFECTS IN F5 RESULTS IN A FORM OF THROMBOPHILIA KNOWN AS APC RESISTANCE (APCR). THE APCR MUTATION IS FOUND IN ABOUT 5% OF THE POPULATION WHICH SUGGEST THAT A SLIGHT THROMBOTIC TENDENCY MAY CONFER SOME ADVANTAGE IN FETAL IMPLANTATION.
- **SIMILARITY:** CONTAINS 3 F5/8 TYPE A DOMAINS; EACH IS COMPOSED OF 2 PLASTOCYANIN-LIKE REPEATS.
- **SIMILARITY:** CONTAINS 2 F5/8 TYPE C DOMAINS.
- **SIMILARITY:** STRONG, TO COAGULATION FACTOR VIII.

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PIR A25897; A25897.
A28028; A28028.
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134400 [\[NCBI / EBI\]](#).
MIM 188055 [\[NCBI / EBI\]](#).
227310 [\[NCBI / EBI\]](#).
227400 [\[NCBI / EBI\]](#).
GeneCards F5.
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IPR000421; FA58_C.
[Graphical view of domain structure.](#)
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PF00754; F5_F8_type_C: 2.
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PS00079; MULTICOPPER_OXIDASE1: 2.
PROSITE PS01285; FA58C_1; 2.
PS01286; FA58C_2; 2.
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BLOCKS P12259.
DOMO P12259.
ProtoMap P12259.
PRESAGE P12259.
DIP P12259.
SWISS-2DPAGE [GET REGION ON 2D PAGE.](#)

Keywords

Blood coagulation; Plasma; Glycoprotein; Calcium; Signal; Zymogen; Repeat; Polymorphism; Disease mutation; Thrombophilia.

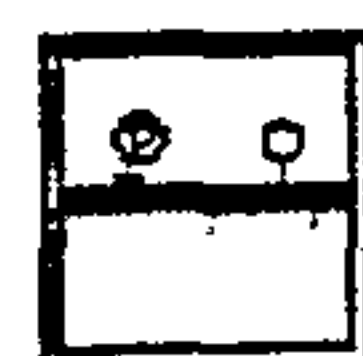
Features

Key	From	To	Length	Description
SIGNAL	1	28	28	
CHAIN	29	2224	2196	COAGULATION FACTOR V.
CHAIN	29	737	709	HEAVY CHAIN.

PEPTIDE	<u>738</u>	<u>1573</u>	836	ACTIVATION PEPTIDE (CONNECTING REGION).
CHAIN	<u>1574</u>	<u>2224</u>	651	LIGHT CHAIN.
DOMAIN	<u>30</u>	<u>329</u>	300	F5/8 TYPE A 1.
DOMAIN	<u>30</u>	<u>193</u>	164	PLASTOCYANIN-LIKE 1.
DOMAIN	<u>203</u>	<u>329</u>	127	PLASTOCYANIN-LIKE 2.
DOMAIN	<u>348</u>	<u>684</u>	337	F5/8 TYPE A 2.
DOMAIN	<u>348</u>	<u>526</u>	179	PLASTOCYANIN-LIKE 3.
DOMAIN	<u>536</u>	<u>684</u>	149	PLASTOCYANIN-LIKE 4.
DOMAIN	<u>692</u>	<u>1573</u>	882	B.
DOMAIN	<u>895</u>	<u>928</u>	34	2 X 17 AA TANDEM REPEATS.
REPEAT	<u>895</u>	<u>911</u>	17	1.
REPEAT	<u>912</u>	<u>928</u>	17	2.
SIMILAR	<u>1135</u>	<u>1148</u>	14	TO 14 AA REPEATS IN BOVINE FA5.
DOMAIN	<u>1185</u>	<u>1463</u>	279	35 X 9 AA TANDEM REPEATS OF [TNP]-L-S-P-D-L-S-Q-T (APPROXIMATE).
REPEAT	<u>1185</u>	<u>1193</u>	9	1.
REPEAT	<u>1194</u>	<u>1202</u>	9	2.
REPEAT	<u>1203</u>	<u>1211</u>	9	3.
REPEAT	<u>1212</u>	<u>1220</u>	9	4.
REPEAT	<u>1221</u>	<u>1229</u>	9	5.
REPEAT	<u>1230</u>	<u>1238</u>	9	6.
REPEAT	<u>1239</u>	<u>1247</u>	9	7.
REPEAT	<u>1248</u>	<u>1256</u>	9	8.
REPEAT	<u>1257</u>	<u>1265</u>	9	9.
REPEAT	<u>1266</u>	<u>1274</u>	9	10.
REPEAT	<u>1275</u>	<u>1283</u>	9	11.
REPEAT	<u>1284</u>	<u>1292</u>	9	12.
REPEAT	<u>1293</u>	<u>1301</u>	9	13.
REPEAT	<u>1302</u>	<u>1310</u>	9	14.
REPEAT	<u>1311</u>	<u>1319</u>	9	15.
REPEAT	<u>1320</u>	<u>1328</u>	9	16.
REPEAT	<u>1329</u>	<u>1337</u>	9	17.
REPEAT	<u>1338</u>	<u>1346</u>	9	18.
REPEAT	<u>1347</u>	<u>1355</u>	9	19.
REPEAT	<u>1356</u>	<u>1364</u>	9	20.
REPEAT	<u>1365</u>	<u>1373</u>	9	21.
REPEAT	<u>1374</u>	<u>1382</u>	9	22.
REPEAT	<u>1383</u>	<u>1391</u>	9	23.
REPEAT	<u>1392</u>	<u>1400</u>	9	24.
REPEAT	<u>1401</u>	<u>1409</u>	9	25.
REPEAT	<u>1410</u>	<u>1418</u>	9	26.
REPEAT	<u>1419</u>	<u>1427</u>	9	27.
REPEAT	<u>1428</u>	<u>1436</u>	9	28.
REPEAT	<u>1437</u>	<u>1445</u>	9	29.
REPEAT	<u>1446</u>	<u>1454</u>	9	30.
REPEAT	<u>1455</u>	<u>1463</u>	9	31.
REPEAT	<u>1464</u>	<u>1472</u>	9	32.
REPEAT	<u>1473</u>	<u>1481</u>	9	33.
REPEAT	<u>1482</u>	<u>1490</u>	9	34.
REPEAT	<u>1493</u>	<u>1501</u>	9	35.
DOMAIN	<u>1578</u>	<u>1907</u>	330	F5/8 TYPE A 3.
DOMAIN	<u>1578</u>	<u>1751</u>	174	PLASTOCYANIN-LIKE 5.
DOMAIN	<u>1761</u>	<u>1907</u>	147	PLASTOCYANIN-LIKE 6.
DOMAIN	<u>1907</u>	<u>2061</u>	155	F5/8 TYPE C 1.
DOMAIN	<u>2066</u>	<u>2221</u>	156	F5/8 TYPE C 2.
SITE	<u>737</u>	<u>738</u>	2	CLEAVAGE (BY THROMBIN).
SITE	<u>1046</u>	<u>1047</u>	2	CLEAVAGE (BY THROMBIN).
SITE	<u>1573</u>	<u>1574</u>	2	CLEAVAGE (BY THROMBIN).
DISULFID	<u>167</u>	<u>193</u>		PROBABLE.



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L -> I (IN REF. 3).
V -> M (IN REF. 1).
T -> A (IN REF. 4).

Length: 2224 AA [This is the length of the unprocessed precursor] Molecular weight: 251699 Da [This is the MW of the unprocessed precursor] CRC64: 470DD8CD00D2303B [This is a checksum on the sequence]

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precursor]
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MFPGCPR LWV LVLGTSWVG WGSQGTEAAQ LRQFYVAAQG ISWSYRREPT NSSLNLSVTS

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      70      80      90      100      110      120
      |      |      |      |      |      |
FKKIVYREYE PYFKKEKPOS TISGLLGPTL YAEVGDIKIV HFKNKADKPL SIHPQGIRYS

     130     140     150     160     170     180
     |     |     |     |     |     |
KLSEGASYLD HTFPAEKMDD AVAPGREYTY EWSISEDSPG THDDPPCLTH IYYSHENLIE

     190     200     210     220     230     240
     |     |     |     |     |     |
DFNSGLIGPL LICKKGTLTE GGTQKTFDKQ IVLLFAVFDE SKWSQSSSL MYTVNGYVNG

     250     260     270     280     290     300
     |     |     |     |     |     |
TMPDITVCAH DHISWHLLGM SSGPELFSIH FNGQVLEQNH HKVSAITLVS ATSTTANMTV

     310     320     330     340     350     360
     |     |     |     |     |     |
GPEGKWIISS LTPKHLQAGM QAYIDIKNCP KKTRNLKKIT REQRRHMKRW EYFIAAEEVI

     370     380     390     400     410     420
     |     |     |     |     |     |
WDYAPVIPAN MDKKYRSQHL DNFSNQIGKH YKKVMTQYE DESFTKHTVN PNMKEDGILG

     430     440     450     460     470     480
     |     |     |     |     |     |
PIIRAQVRDT LKIVFKNMAS RPYSIYPHGV TFSPEYDEVN SSFTSGRNNT MIRAVQPGET

     490     500     510     520     530     540
     |     |     |     |     |     |
YTYKWNILEF DEPTENDAQC LTRPYSDVD IMRDIASGLI GLLICKSRS LDRRGIQRAA

     550     560     570     580     590     600
     |     |     |     |     |     |
DIEQQAVFAV FDENKSWYLE DNINKFCENP DEVKRDDPKF YESNIMSTIN GYVPESITTL

     610     620     630     640     650     660
     |     |     |     |     |     |
GFCFDDTVQW HFCSVGTQNE ILTIHFTGHS FIYGRHEDT LTLFPMRGES VVTMDNVGT

     670     680     690     700     710     720
     |     |     |     |     |     |
WMLTSMNSSP RSKKLRLKFR DVKICIPDDE DSYEIFEPPE STVMATRKMH DRLEPEDEES

     730     740     750     760     770     780
     |     |     |     |     |     |
DADYDYNRL AAALGIRSFR NSSLNQEEEE FNLTALALEN GTEFVSSNTD IIVGSNYSSP

     790     800     810     820     830     840
     |     |     |     |     |     |
SNISKFTVNN LAEPQKAPSH QQATTAGSPL RHLIGKNSVL NSSTAESSP YSEDPIEDPL

     850     860     870     880     890     900
     |     |     |     |     |     |
QPDVTGIRLL SLGAGEFKSQ EHAKHKGPKV ERDQAAKHF SWMKLLAHKV GRHLSQDTGS

     910     920     930     940     950     960
     |     |     |     |     |     |
PSGMRPWEDL PSQDTGSPSR MRPWKDPPSD LLLKQSNSS KILVGRWHLA SEKGSYEIIQ

     970     980     990     1000     1010     1020
     |     |     |     |     |     |
DTDEDTAVNN WLISPQNASR AWGESTPLAN KPGKQSGHPK FPRVRHKSLO VRQDGGKSRL

    1030    1040    1050    1060    1070    1080
     |     |     |     |     |     |
KKSQFLIKTR KKKKEKHTHH APLSPRTFHP LRSEAYNTFS ERRLKHSVLV HKSNETSLPT

    1090    1100    1110    1120    1130    1140
     |     |     |     |     |     |
DLNQTLPSMD FGWIASLPDH NQNSSNDTGQ ASCPPGLYQT VPPEEHYQTF PIQDPDQMS

    1150    1160    1170    1180    1190    1200
     |     |     |     |     |     |
TSDPSHRSSS PELSEMLEYD RSHKSFTPTDI SQMSPSSEHE VWQTVISPD L SQVTLSPELS

    1210    1220    1230    1240    1250    1260
     |     |     |     |     |     |
QTNLSPDLSH TTLSPELIQR NLSPALGQMP ISPDLSHTTL SPDLSHHTLS LDLSQTNLSP

    1270    1280    1290    1300    1310    1320
     |     |     |     |     |     |
ELSQTNLSPA LGQMPLSPDL SHTTSLDIFS QTNLSPELSH MTLSPELSQT NLSPALGQMP

    1330    1340    1350    1360    1370    1380
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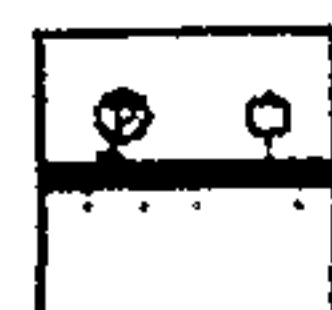
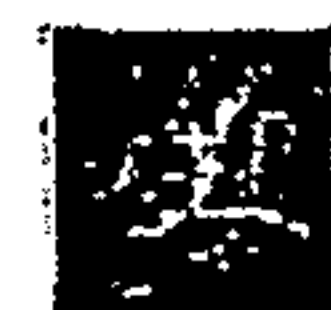
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ISPDLSHHTL SLDFSQTNLS PELSQTNLSP ALGQMPLSPD PSHTTSLDL SQTNLSPELS
      |      |      |      |      |      |
      1390      1400      1410      1420      1430      1440
      |      |      |      |      |      |
QTNLSPDLSE MPLFADLSQI PLTPDLQMT LSPDLGETDL SPNFGQMSLS PDLSQVTLSP
      |      |      |      |      |      |
      1450      1460      1470      1480      1490      1500
      |      |      |      |      |      |
DISDTLLLPD LSQISPPPD LQIFYPSESS QSLLLQEFNE SFPYPDLGQM PSPSSPTLND
      |      |      |      |      |      |
      1510      1520      1530      1540      1550      1560
      |      |      |      |      |      |
TFLSKEFNPL VIVGLSKDGT DYIEIIPKEE VQSEDDYAE IDYVPYDDPY KTDVRTNINS
      |      |      |      |      |      |
      1570      1580      1590      1600      1610      1620
      |      |      |      |      |      |
SRDPDNIAAW YLRNNGNRR NYIIAAEEIS WDYSEFVQRE TDIEDSDDIP EDTTYKKVVF
      |      |      |      |      |      |
      1630      1640      1650      1660      1670      1680
      |      |      |      |      |      |
RKYLDSTFTK RDPRGEYEEH LGILGPIIRA EVDDVIQVRF KNLASRPYSL HAHGLSYEKS
      |      |      |      |      |      |
      1690      1700      1710      1720      1730      1740
      |      |      |      |      |      |
SEGKTYEDDS PEWFKEDNAV QPNSSYTYVW HATERSGPES PGSACRAWAY YSAVNPEKDI
      |      |      |      |      |      |
      1750      1760      1770      1780      1790      1800
      |      |      |      |      |      |
HSLGIGPLLI CQKGILHKDS NMPVDMREFV LLFMTFDEKK SWYYEKKSRS SWRLTSSEMK
      |      |      |      |      |      |
      1810      1820      1830      1840      1850      1860
      |      |      |      |      |      |
KSHEFHAING MIYSLPGLKM YEQEWVRLHL LNIGGSQDIH VVHFHGQTLL ENGNKQHQLG
      |      |      |      |      |      |
      1870      1880      1890      1900      1910      1920
      |      |      |      |      |      |
VWPLLPGSEFK TLEMKASKPG WLLNTEVEGE NQRAGMQTPF LIMDRDCRMP MGLSTGIISD
      |      |      |      |      |      |
      1930      1940      1950      1960      1970      1980
      |      |      |      |      |      |
SQIKASEFLG YWEPRLARLN NGGSYNAWSV EKLAAEFASK PWIQVDMQKE VIITGIQTQG
      |      |      |      |      |      |
      1990      2000      2010      2020      2030      2040
      |      |      |      |      |      |
AKHYLKSCYT TEFYVAYSSN QINWQIFKGN STRNVMYFNG NSDASTIKEN QFDPPIVARY
      |      |      |      |      |      |
      2050      2060      2070      2080      2090      2100
      |      |      |      |      |      |
IRISPTRAYN RPTLRLELQG CEVNGCSTPL GMENGKIENK QITASSFKKS WWDYWEFPR
      |      |      |      |      |      |
      2110      2120      2130      2140      2150      2160
      |      |      |      |      |      |
ARLNAQGRVN AWQAKANNK QWLEIDLLKI KKITAIITQG CKSLSEMYV KSYTIHYSEQ
      |      |      |      |      |      |
      2170      2180      2190      2200      2210      2220
      |      |      |      |      |      |
GVEWKPYRLK SSMVDKIFEG NTNTKGHVKN FFPPIISRF IRVIPKTWNQ SITLRLELFG

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General information about the entry

Entry name **THRB_HUMAN**
Primary accession number **P00734**
Secondary accession numbers None
Entered in SWISS-PROT in Release 01, July 1986
Sequence was last modified in Release 13, January 1990
Annotations were last modified in Release 40, October 2000

Name and origin of the protein

Protein name **PROTHROMBIN [Precursor]**
Synonyms **EC 3.4.21.5**
COAGULATION FACTOR II
Gene name **F2**
From **Homo sapiens (Human) [TaxID: 9606]**
Taxonomy **Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.**

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Comments

- **FUNCTION:** THROMBIN, WHICH CLEAVES BONDS AFTER ARG & LYS, CONVERTS FIBRINOGEN TO FIBRIN AND ACTIVATES FACTORS V, VII, VIII, XIII. AND, IN COMPLEX WITH THROMBOMODULIN, PROTEIN C.
- **SUBCELLULAR LOCATION:** EXTRACELLULAR.
- **TISSUE SPECIFICITY:** SYNTHESIZED IN THE LIVER; FOUND IN PLASMA.
- **PTM:** THE GAMMA-CARBOXYGLUTAMYL RESIDUES, WHICH BIND CALCIUM IONS, RESULT FROM THE CARBOXYLATION OF GLUTAMYL RESIDUES BY A MICROSOMAL ENZYME, THE VITAMIN K-DEPENDENT CARBOXYLASE. THE MODIFIED RESIDUES ARE NECESSARY FOR THE CA-DEPENDENT INTERACTION WITH A NEGATIVELY CHARGED PHOSPHOLIPID SURFACE, WHICH IS ESSENTIAL FOR THE CONVERSION OF PROTHROMBIN TO THROMBIN.
- **DISEASE:** DEFECTS IN F2 ARE THE CAUSE OF VARIOUS FORMS OF DYSPROTHROMBINEMIA.
- **MISCELLANEOUS:** PROTHROMBIN IS ACTIVATED ON THE SURFACE OF A PHOSPHOLIPID MEMBRANE THAT BINDS THE AMINO END OF PROTHROMBIN & FACTORS VA & XA IN CA-DEPENDENT INTERACTIONS; FACTOR XA REMOVES THE ACTIVATION PEPTIDE & CLEAVES THE REMAINING PART INTO LIGHT & HEAVY CHAINS. THE ACTIVATION PROCESS STARTS SLOWLY BECAUSE FACTOR V ITSELF HAS TO BE ACTIVATED BY THE INITIAL, SMALL AMOUNTS OF THROMBIN.
- **MISCELLANEOUS:** IT IS NOT KNOWN WHETHER 1 OR 2 SMALLER ACTIVATION PEPTIDES, WITH ADDITIONAL CLEAVAGE AFTER 314-ARG, ARE RELEASED IN NATURAL BLOOD CLOTTING.
- **MISCELLANEOUS:** THROMBIN CAN ITSELF CLEAVE THE AMINO TERMINAL FRAGMENT (FRAGMENT 1) OF THE PROTHROMBIN, PRIOR TO ITS ACTIVATION BY FACTOR XA.
- **MISCELLANEOUS:** THE CLEAVAGE AFTER R-198, OBSERVED IN VITRO, DOES NOT OCCUR IN PLASMA.
- **SIMILARITY:** CONTAINS 2 KRINGLE DOMAINS.
- **SIMILARITY:** BELONGS TO PEPTIDASE FAMILY S1; ALSO KNOWN AS THE TRYPSIN FAMILY.

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1BB0; 27-APR-99. [[ExPASy](#) / [RCSB](#)]

MEROPS [S01.217](#); -.
SWISS-2DPAGE [P00734](#); HUMAN.
MIM [176930](#) [[NCBI](#) / [EBI](#)].
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[IPR001314](#); Chymotrypsin.
[IPR002383](#); GLA blood.
InterPro [IPR000001](#); Kringle.
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PRINTS [PR00001](#); GLABLOOD.
[PR00018](#); KRINGLE.
[PR00722](#); CHYMOTRYPSIN.
SMART [SM00069](#); GLA; 1.
[SM00130](#); KR; 2.
[SM00020](#); Tryp SPc; 1.
PROSITE [PS00011](#); GLU CARBOXYLATION; 1.
[PS00021](#); KRINGLE 1; 2.
[PS00070](#); KRINGLE 2; 2.
[PS00240](#); TRYPSIN DOM; 1.
[PS00134](#); TRYPSIN HIS; 1.
[PS00135](#); TRYPSIN SER; 1.
ProDom [\[Domain structure / List of seq. sharing at least 1 domain\]](#).
BLOCKS [P00734](#).
DOMO [P00734](#).
ProtoMap [P00734](#).
PRESAGE [P00734](#).
DIP [P00734](#).

Keywords

Blood coagulation; Plasma; Calcium-binding; Glycoprotein; Duplication; Vitamin K; Zymogen; Gamma-carboxyglutamic acid; Acute phase; Liver; Hydrolase; Serine protease; Kringle; Signal; 3D-structure; Disease mutation.

Features

Key	From	To	Length	Description
SIGNAL	<u>1</u>	<u>24</u>	24	POTENTIAL.
PROPEP	<u>25</u>	<u>43</u>	19	

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CHAIN	<u>44</u>	<u>622</u>	579	PROTHROMBIN.
PEPTIDE	<u>44</u>	<u>198</u>	155	ACTIVATION PEPTIDE (FRAGMENT 1).
PEPTIDE	<u>199</u>	<u>327</u>	129	ACTIVATION PEPTIDE (FRAGMENT 2).
CHAIN	<u>328</u>	<u>363</u>	36	THROMBIN LIGHT CHAIN (A).
CHAIN	<u>364</u>	<u>622</u>	259	THROMBIN HEAVY CHAIN (B).
DOMAIN	<u>108</u>	<u>186</u>	79	KRINGLE 1.
DOMAIN	<u>213</u>	<u>291</u>	79	KRINGLE 2.
DOMAIN	<u>364</u>	<u>622</u>	259	SERINE PROTEASE.
SITE	<u>198</u>	<u>199</u>	2	CLEAVAGE (BY THROMBIN).
SITE	<u>327</u>	<u>328</u>	2	CLEAVAGE (BY FACTOR XA).
SITE	<u>363</u>	<u>364</u>	2	CLEAVAGE (BY FACTOR XA).
ACT_SITE	<u>406</u>	<u>406</u>		CHARGE RELAY SYSTEM.
ACT_SITE	<u>462</u>	<u>462</u>		CHARGE RELAY SYSTEM.
ACT_SITE	<u>568</u>	<u>568</u>		CHARGE RELAY SYSTEM.
MOD_RES	<u>49</u>	<u>49</u>		GAMMA-CARBOXYGLUTAMIC ACID.
MOD_RES	<u>50</u>	<u>50</u>		GAMMA-CARBOXYGLUTAMIC ACID.
MOD_RES	<u>57</u>	<u>57</u>		GAMMA-CARBOXYGLUTAMIC ACID.
MOD_RES	<u>59</u>	<u>59</u>		GAMMA-CARBOXYGLUTAMIC ACID.
MOD_RES	<u>62</u>	<u>62</u>		GAMMA-CARBOXYGLUTAMIC ACID.
MOD_RES	<u>63</u>	<u>63</u>		GAMMA-CARBOXYGLUTAMIC ACID.
MOD_RES	<u>68</u>	<u>68</u>		GAMMA-CARBOXYGLUTAMIC ACID.
MOD_RES	<u>69</u>	<u>69</u>		GAMMA-CARBOXYGLUTAMIC ACID.
MOD_RES	<u>72</u>	<u>72</u>		GAMMA-CARBOXYGLUTAMIC ACID.
MOD_RES	<u>75</u>	<u>75</u>		GAMMA-CARBOXYGLUTAMIC ACID.
CARBOHYD	<u>121</u>	<u>121</u>		N-LINKED (GLCNAC...).
CARBOHYD	<u>143</u>	<u>143</u>		N-LINKED (GLCNAC...).
CARBOHYD	<u>416</u>	<u>416</u>		N-LINKED (GLCNAC...).
DISULFID	<u>60</u>	<u>65</u>		
DISULFID	<u>90</u>	<u>103</u>		
DISULFID	<u>108</u>	<u>186</u>		
DISULFID	<u>129</u>	<u>169</u>		
DISULFID	<u>157</u>	<u>181</u>		
DISULFID	<u>213</u>	<u>291</u>		
DISULFID	<u>234</u>	<u>274</u>		
DISULFID	<u>262</u>	<u>286</u>		
DISULFID	<u>336</u>	<u>482</u>		INTERCHAIN.
DISULFID	<u>391</u>	<u>407</u>		
DISULFID	<u>536</u>	<u>550</u>		BY SIMILARITY.
DISULFID	<u>564</u>	<u>594</u>		BY SIMILARITY.
VARIANT	<u>200</u>	<u>200</u>		E -> K (IN TYPE 3; DYSPROTHROMBINEMIA). /FTid=VAR_006711.
VARIANT	<u>314</u>	<u>314</u>		R -> C (IN BARCELONA/MADRID; DYSPROTHROMBINEMIA). /FTid=VAR_006712.
VARIANT	<u>314</u>	<u>314</u>		R -> H (IN PALUA-1; DYSPROTHROMBINEMIA). /FTid=VAR_006713.
VARIANT	<u>380</u>	<u>380</u>		M -> T (IN HIMI-1; DYSPROTHROMBINEMIA). /FTid=VAR_006714.
VARIANT	<u>425</u>	<u>425</u>		R -> C (IN QUICK-1; DYSPROTHROMBINEMIA). /FTid=VAR_006715.
VARIANT	<u>431</u>	<u>431</u>		R -> H (IN HIMI-2; DYSPROTHROMBINEMIA). /FTid=VAR_006716.
VARIANT	<u>461</u>	<u>461</u>		R -> W (IN TOKUSHIMA; DYSPROTHROMBINEMIA). /FTid=VAR_006717.
VARIANT	<u>509</u>	<u>509</u>		E -> A (IN SALAKTA/FRANKFURT). /FTid=VAR_006718.
VARIANT	<u>601</u>	<u>601</u>		G -> V (IN QUICK-2; DYSPROTHROMBINEMIA). /FTid=VAR_006719.
CONFLICT	<u>119</u>	<u>119</u>		H -> N (IN REF. 3).

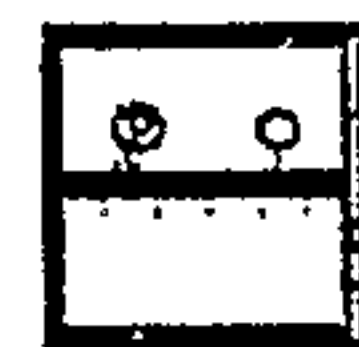
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CONFLICT	<u>121</u>	<u>121</u>	
CONFLICT	<u>164</u>	<u>164</u>	
CONFLICT	<u>164</u>	<u>164</u>	
CONFLICT	<u>176</u>	<u>176</u>	
CONFLICT	<u>183</u>	<u>183</u>	
CONFLICT	<u>194</u>	<u>195</u>	
CONFLICT	<u>308</u>	<u>308</u>	
CONFLICT	<u>335</u>	<u>335</u>	
CONFLICT	<u>349</u>	<u>349</u>	
CONFLICT	<u>369</u>	<u>369</u>	
CONFLICT	<u>398</u>	<u>398</u>	
CONFLICT	<u>414</u>	<u>414</u>	
CONFLICT	<u>485</u>	<u>485</u>	
CONFLICT	<u>494</u>	<u>494</u>	
CONFLICT	<u>504</u>	<u>504</u>	
CONFLICT	<u>509</u>	<u>509</u>	
CONFLICT	<u>511</u>	<u>511</u>	
CONFLICT	<u>514</u>	<u>514</u>	
CONFLICT	<u>529</u>	<u>530</u>	
CONFLICT	<u>532</u>	<u>532</u>	
TURN	<u>336</u>	<u>337</u>	2
TURN	<u>340</u>	<u>346</u>	7
TURN	<u>350</u>	<u>351</u>	2
HELIX	<u>352</u>	<u>356</u>	5
TURN	<u>357</u>	<u>358</u>	2
STRAND	<u>365</u>	<u>365</u>	1
STRAND	<u>368</u>	<u>369</u>	2
TURN	<u>372</u>	<u>373</u>	2
TURN	<u>376</u>	<u>377</u>	2
STRAND	<u>378</u>	<u>383</u>	6
TURN	<u>384</u>	<u>387</u>	4
STRAND	<u>388</u>	<u>395</u>	8
STRAND	<u>400</u>	<u>403</u>	4
HELIX	<u>405</u>	<u>407</u>	3
TURN	<u>408</u>	<u>408</u>	1
STRAND	<u>409</u>	<u>410</u>	2
HELIX	<u>411</u>	<u>413</u>	3
TURN	<u>414</u>	<u>414</u>	1
STRAND	<u>415</u>	<u>416</u>	2
HELIX	<u>419</u>	<u>421</u>	3
STRAND	<u>422</u>	<u>426</u>	5
TURN	<u>436</u>	<u>438</u>	3
STRAND	<u>440</u>	<u>449</u>	10
TURN	<u>451</u>	<u>452</u>	2
STRAND	<u>454</u>	<u>454</u>	1
TURN	<u>455</u>	<u>458</u>	4
STRAND	<u>460</u>	<u>460</u>	1
STRAND	<u>464</u>	<u>468</u>	5
STRAND	<u>475</u>	<u>475</u>	1
TURN	<u>476</u>	<u>477</u>	2
STRAND	<u>478</u>	<u>478</u>	1
STRAND	<u>482</u>	<u>482</u>	1
HELIX	<u>486</u>	<u>492</u>	7
TURN	<u>495</u>	<u>496</u>	2
STRAND	<u>498</u>	<u>503</u>	6
TURN	<u>510</u>	<u>511</u>	2
STRAND	<u>524</u>	<u>530</u>	7
HELIX	<u>533</u>	<u>538</u>	6
TURN	<u>539</u>	<u>539</u>	1
TURN	<u>546</u>	<u>547</u>	2

N -> S (IN REF. 3).
T -> N (IN REF. 2).
T -> I (IN REF. 3).
V -> A (IN REF. 3).
I -> T (IN REF. 3).
AM -> MV (IN REF. 3).
D -> DEE (IN REF. 3).
D -> N (IN REF. 4).
D -> N (IN REF. 4).
D -> N (IN REF. 4).
D -> N (IN REF. 4).
D -> N (IN REF. 4).
D -> N (IN REF. 4).
Q -> G (IN REF. 4).
W -> Y (IN REF. 4).
E -> S (IN REF. 4).
W -> V (IN REF. 4).
N -> D (IN REF. 4).
PI -> AL (IN REF. 4).
E -> Q (IN REF. 4).

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table
viewer](#)

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STRAND	<u>548</u>	<u>551</u>	4
TURN	<u>555</u>	<u>557</u>	3
STRAND	<u>562</u>	<u>562</u>	1
TURN	<u>565</u>	<u>566</u>	2
TURN	<u>568</u>	<u>569</u>	2
STRAND	<u>571</u>	<u>575</u>	5
TURN	<u>577</u>	<u>579</u>	3
STRAND	<u>582</u>	<u>590</u>	9
TURN	<u>597</u>	<u>598</u>	2
STRAND	<u>601</u>	<u>605</u>	5
HELIX	<u>606</u>	<u>617</u>	12
TURN	<u>618</u>	<u>618</u>	1

Sequence information

Length: 622 AA [This is the length of the unprocessed precursor] Molecular weight: 70036 Da [This is the MW of the unprocessed precursor] CRC64: 8A25E1DA88208FCF [This is a checksum on the sequence]

10	20	30	40	50	60
MAHVRGLQLP	GCLALAAALCS	LVHSQHVFLA	PQQARSLQOR	VRRANTFLEE	VRKGNLEREC
70	80	90	100	110	120
VEETCSYEEA	FEALESSTAT	DVFWAKYTAC	ETARTPRDKL	AACLEGNCAE	GLGTNYRGHV
130	140	150	160	170	180
NITRSGIECQ	LWRSRYPHKP	EINSTTHPGA	DLQENFCRNP	DSSTGPGWCY	TTDPTVRRQE
190	200	210	220	230	240
CSIPVCGQDQ	VTVAMTPRSE	GSSVNLSPPL	EQCVPDGRGQ	YQRLAVTTH	GLPCLAWASA
250	260	270	280	290	300
QAKALSKHQD	FNSAVQLVEN	FCRNPDGDEE	GVWCYVAGKP	GDFGYCDLNY	CEEAVEEETG
310	320	330	340	350	360
DGLDEDSdra	IEGRTATSEY	QTFNPRFTFG	SGEADCGLRP	LFEKKSLEDK	TERELLESYI
370	380	390	400	410	420
DGRIVEGSDA	EIGMSPWQVM	LFRKSPQELL	CGASLISDRW	VLTAAHCLLY	PPWDKNFTEN
430	440	450	460	470	480
DLLVRIGKHS	RTRYERNIEK	ISMLEKIYIH	PRYNWRENLD	RDIALMKLKK	PVAFSDYIHP
490	500	510	520	530	540
VCLPDRETAA	SLLQAGYKGR	VTGWGNLKET	WTANVGKGQP	SVLQVNLPI	VERPVCKDST
550	560	570	580	590	600
RIRITDNMFC	AGYKPDEGKR	GDACEGDSGG	PFVMKSPFNN	RWYQMGIVSW	GEGCDRDGKY
610	620				
GFYTHVFRLLK	KWIKVIDQF	GE			

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[\[General\]](#) [\[Name and origin\]](#) [\[References\]](#) [\[Comments\]](#) [\[Cross-references\]](#) [\[Keywords\]](#) [\[Features\]](#)
[\[Sequence\]](#) [\[Tools\]](#)

General information about the entry

Entry name	FA12_HUMAN
Primary accession number	P00748
Secondary accession number	P78339
Entered in SWISS-PROT in	Release 01, July 1986
Sequence was last modified in	Release 12, October 1989
Annotations were last modified in	Release 40, October 2000

Name and origin of the protein

Protein name	COAGULATION FACTOR XII [Precursor]
Synonyms	EC 3.4.21.38 HAGEMAN FACTOR HAF
Gene name	F12
From	Homo sapiens (Human) [TaxID: 9606]
Taxonomy	Eukaryota ; Metazoa ; Chordata ; Craniata ; Vertebrata ; Euteleostomi ; Mammalia ; Eutheria ; Primates ; Catarrhini ; Hominidae ; Homo .

References

FIG. 11

- [1] SEQUENCE FROM NUCLEIC ACID.
MEDLINE=88007593; PubMed=2888762; [NCBI, ExPASy, EBI, Israel, Japan]
Cool D.E., McGillivray R.T.A.;
"Characterization of the human blood coagulation factor XII gene. Intron/exon gene organization and analysis of the 5'-flanking region.";
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MEDLINE=86176794; PubMed=3754331; [NCBI, ExPASy, EBI, Israel, Japan]
Tripodi M., Citarella F., Guida S., Galeffi P., Fantoni A., Cortese R.;
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MEDLINE=86033830; PubMed=3877053; [NCBI, ExPASy, EBI, Israel, Japan]
Cool D.E., Edgell C.-J.S., Louie G.V., Zoller M.J., Braver G.D., McGillivray R.T.A.;
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MEDLINE=86216049; PubMed=3011063; [NCBI, ExPASy, EBI, Israel, Japan]
Que B.G., Davie E.W.;
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MEDLINE=83291041; PubMed=6604055; [NCBI, ExPASy, EBI, Israel, Japan]
Fujikawa K., McMullen B.A.;
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- [7] SEQUENCE OF 561-615 FROM NUCLEIC ACID.
TISSUE=Blood;
MEDLINE=96133302; PubMed=8528215; [NCBI, ExPASy, EBI, Israel, Japan]
Schloesser M., Hofferbert S., Bartz U., Lutze G., Lammle B., Engel W.;
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- [8] VARIANT WASHINGTON DC.
MEDLINE=90046788; PubMed=2510163; [NCBI, ExPASy, EBI, Israel, Japan]
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- [9] VARIANT LOCARNO.
MEDLINE=94325559; PubMed=8049433; [NCBI, ExPASy, EBI, Israel, Japan]
Hovenga J.K., Schaller J., Stricker H., Willemin W.A., Furlan M., Laemmle B.;
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MEDLINE=92184750; PubMed=1544894; [NCBI, ExPASy, EBI, Israel, Japan]
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"O-linked fucose is present in the first epidermal growth factor domain of factor XII but not protein C.";
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Comments

- **FUNCTION:** FACTOR XII IS A SERUM GLYCOPROTEIN THAT PARTICIPATES IN THE INITIATION OF BLOOD COAGULATION, FIBRINOLYSIS, AND THE GENERATION OF BRADYKININ AND ANGIOTENSIN.
- **CATALYTIC ACTIVITY:** CLEAVES SELECTIVELY ARG-|-ILE BONDS AND ACTIVATES COAGULATION FACTORS VII AND XI.
- **PTM:** O- AND N-GLYCOSYLATED.

- **DISEASE:** DEFECTS IN F12 DO NOT CAUSE ANY CLINICAL SYMPTOMS. THE SOLE EFFECT IS THAT WHOLE-BLOOD CLOTTING TIME IS PROLONGED.
- **MISCELLANEOUS:** FACTOR XII, PREKALLIKREIN, AND HMW KININOGEN FORM A COMPLEX BOUND TO AN ANIONIC SURFACE. PREKALLIKREIN IS CLEAVED BY FACTOR XII TO FORM KALLIKREIN, WHICH THEN CLEAVES FACTOR XII FIRST TO ALPHA-FACTOR XIIA AND THEN TO BETA-FACTOR XIIA. ALPHA-FACTOR XIIA ACTIVATES FACTOR XI TO FACTOR XIA.
- **SIMILARITY:** CONTAINS 2 EGF-LIKE DOMAINS.
- **SIMILARITY:** CONTAINS 1 FIBRONECTIN TYPE I DOMAIN.
- **SIMILARITY:** CONTAINS 1 FIBRONECTIN TYPE II DOMAIN.
- **SIMILARITY:** CONTAINS 1 KRINGLE DOMAIN.
- **SIMILARITY:** BELONGS TO PEPTIDASE FAMILY S1; ALSO KNOWN AS THE TRYPSIN FAMILY.

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Cross-references

EMBL	M31315; AAA70225.1; -. [EMBL / GenBank / DDBJ] [CoDingSequence]
	M11723; AAA51986.1; -. [EMBL / GenBank / DDBJ] [CoDingSequence]
	M17466; AAB59490.1; -. [EMBL / GenBank / DDBJ] [CoDingSequence]
	M17464; AAB59490.1; JOINED. [EMBL / GenBank / DDBJ] [CoDingSequence]
	M17465; AAB59490.1; JOINED. [EMBL / GenBank / DDBJ] [CoDingSequence]
	M13147; AAA70224.1; -. [EMBL / GenBank / DDBJ] [CoDingSequence]
	U71274; AAB51203.1; -. [EMBL / GenBank / DDBJ] [CoDingSequence]
PIR	A29411; KFHU12.
HSSP	P00763; 1DPO. [HSSP ENTRY / PDB]
MEROPS	S01.211; -. [MEROPS]
MIM	234000 [NCBI / EBI].
GeneCards	F12.
InterPro	IPR001314: Chymotrypsin.
	IPR000561: EGF-like.
	IPR000562: FN Type II.
	IPR000001: Kringle.
	IPR001254: Trypsin.
	IPR000083: fibronectin type 1.
	Graphical view of domain structure.
Pfam	PF00008: EGF; 2.
	PF00039: fn1; 1.
	PF00040: fn2; 1.
	PF00051: kringle; 1.
	PF00089: trypsin; 1.
PRINTS	PR00013: FNTYPEII.
	PR00018: KRINGLE.
	PR00722: CHYMOTRYPSIN.
ProDom	PD000995; -. 1.
	[Domain structure / List of seq. sharing at least 1 domain].
SMART	SM00181: EGF; 2.
	SM00058: FN1; 1.
	SM00059: FN2; 1.
	SM00130: KR; 1.
	SM00020: Tryp_SPC; 1.
	PS00022: EGF_1; 2.
	PS01186: EGF_2; 1.
PROSITE	PS01253: FIBRONECTIN_1; 1.
	PS00023: FIBRONECTIN_2; 1.
	PS00021: KRINGLE_1; 1.
	PS50070: KRINGLE_2; 1.
	PS50240: TRYPSIN_DOM; 1.
	PS00134: TRYPSIN_HIS; 1.
BLOCKS	PS00135: TRYPSIN_SER; 1.
	P00748.

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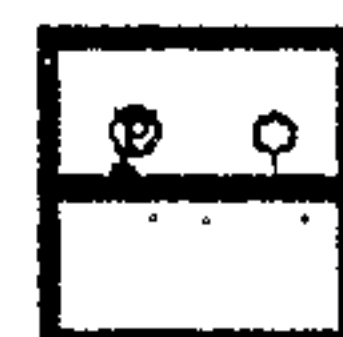
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<http://www.expasy.ch/cgi-bin/sprot-search-de>D0MU P00748.ProtoMap P00748.PRESAGE P00748.DIP P00748.SWISS-2DPAGE GET REGION ON 2D PAGE.**Keywords**

Glycoprotein; Blood coagulation; Plasma; Kringle; Serine protease; Hydrolase; Fibrinolysis;
Signal; EGF-like domain; Repeat; Zymogen; Disease mutation.

Features

Key	From	To	Length	Description
SIGNAL	<u>1</u>	<u>19</u>	19	
CHAIN	<u>20</u>	<u>372</u>	353	ALPHA-FACTOR XIIA HEAVY CHAIN.
CHAIN	<u>373</u>	<u>615</u>	243	ALPHA-FACTOR XIIA LIGHT CHAIN.
CHAIN	<u>354</u>	<u>362</u>	9	BETA-FACTOR XIIA PART 1.
CHAIN	<u>373</u>	<u>615</u>	243	BETA-FACTOR XIIA PART 2.
DOMAIN	<u>47</u>	<u>88</u>	42	FIBRONECTIN TYPE-II.
DOMAIN	<u>94</u>	<u>131</u>	38	EGF-LIKE 1.
DOMAIN	<u>133</u>	<u>173</u>	41	FIBRONECTIN TYPE-I.
DOMAIN	<u>174</u>	<u>210</u>	37	EGF-LIKE 2.
DOMAIN	<u>217</u>	<u>295</u>	79	KRINGLE.
DOMAIN	<u>296</u>	<u>349</u>	54	PRO-RICH.
DOMAIN	<u>373</u>	<u>615</u>	243	SERINE PROTEASE.
CARBOHYD	<u>109</u>	<u>109</u>		O-LINKED (FUC).
CARBOHYD	<u>249</u>	<u>249</u>		N-LINKED (GLCNAC...).
CARBOHYD	<u>299</u>	<u>299</u>		O-LINKED (POTENTIAL).
CARBOHYD	<u>305</u>	<u>305</u>		O-LINKED (POTENTIAL).
CARBOHYD	<u>308</u>	<u>308</u>		O-LINKED (POTENTIAL).
CARBOHYD	<u>328</u>	<u>328</u>		O-LINKED (POTENTIAL).
CARBOHYD	<u>329</u>	<u>329</u>		O-LINKED (POTENTIAL).
CARBOHYD	<u>337</u>	<u>337</u>		O-LINKED (POTENTIAL).
ACT_SITE	<u>412</u>	<u>412</u>		CHARGE RELAY SYSTEM (BY SIMILARITY).
ACT_SITE	<u>461</u>	<u>461</u>		CHARGE RELAY SYSTEM (BY SIMILARITY).
ACT_SITE	<u>563</u>	<u>563</u>		CHARGE RELAY SYSTEM (BY SIMILARITY).
DISULFID	<u>98</u>	<u>110</u>		BY SIMILARITY.
DISULFID	<u>104</u>	<u>119</u>		BY SIMILARITY.
DISULFID	<u>121</u>	<u>130</u>		BY SIMILARITY.
DISULFID	<u>135</u>	<u>163</u>		BY SIMILARITY.
DISULFID	<u>161</u>	<u>170</u>		BY SIMILARITY.
DISULFID	<u>178</u>	<u>189</u>		BY SIMILARITY.
DISULFID	<u>183</u>	<u>198</u>		BY SIMILARITY.
DISULFID	<u>200</u>	<u>209</u>		BY SIMILARITY.
DISULFID	<u>217</u>	<u>295</u>		BY SIMILARITY.
DISULFID	<u>238</u>	<u>277</u>		BY SIMILARITY.
DISULFID	<u>266</u>	<u>290</u>		BY SIMILARITY.
DISULFID	<u>359</u>	<u>486</u>		BY SIMILARITY.
DISULFID	<u>397</u>	<u>413</u>		BY SIMILARITY.
DISULFID	<u>405</u>	<u>475</u>		BY SIMILARITY.
DISULFID	<u>436</u>	<u>439</u>		BY SIMILARITY.
DISULFID	<u>500</u>	<u>569</u>		BY SIMILARITY.
DISULFID	<u>532</u>	<u>548</u>		BY SIMILARITY.
DISULFID	<u>559</u>	<u>590</u>		BY SIMILARITY.
VARIANT	<u>372</u>	<u>372</u>		R -> P (IN LOCARNO; INACTIVE). /FTid=VAR_006623.
VARIANT	<u>590</u>	<u>590</u>		C -> S (IN WASHINGTON DC; INACTIVE). /FTid=VAR_006624.
CONFLICT	<u>333</u>	<u>333</u>		P -> S (IN REF. 3).

[Feature aligner](#)[Feature table viewer](#)

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<http://www.expasy.ch/cgi-bin/sprot-search-de>

CONFLICT 319 319 A -> G (IN REF. 4).
CONFLICT 589 589 G -> R (IN REF. 7).

Sequence information

Length: **615 AA** [This is the length of the unprocessed precursor] Molecular weight: **67818 Da** [This is the MW of the unprocessed precursor] CRC64: **1EB3D3EAA7BAAE9A** [This is a checksum on the sequence]

10	20	30	40	50	60
MRALLLLGFL	LVSLESTLSI	PPWEAPKEHK	YKAEHTVVL	TVTGEPCHP	FQYHRQLYHK
70	80	90	100	110	120
CTHKGRPGPQ	PWCATTPNFD	QDQRWGYCLE	PKKVKDHCSK	HSPCQKGGTC	VNMPSGPHCL
130	140	150	160	170	180
CPQHLLTGNHC	QKEKCFEPQL	LRFFHKNEIW	YRTEQAAVAR	CQCKGPDHAC	QRLASQACRT
190	200	210	220	230	240
NPCLHGGRCL	EVEGHRLLCH	PVGYTGPFCD	VDTKASCYDG	RGLSYRGLAR	TTLSGAPCQP
250	260	270	280	290	300
WASEATYRNV	TAEQARNWGL	GGHAFCRNP	NDIRPWCFVL	NRDRLSWEYC	DLAQCQTPTQ
310	320	330	340	350	360
AAPPTFVSPR	LHVPLMPAQ	APPKPQPTTR	TPPQSQTPGA	LPAKREQPPS	LTRNGPLSCG
370	380	390	400	410	420
QRLRKSLSM	TRVVGGLVAL	RGHPYIAAL	YWGHSFCAGS	LIAPCWVLT	AHCLQDRPAP
430	440	450	460	470	480
EDLTVVLGQE	RRNHSCEPCQ	TLAVRSYRLH	EAFSPVSYQH	DLALLRLQED	ADGSCALLSP
490	500	510	520	530	540
YVQPVCLPSG	AARPSETTLC	QVAGWGHQFE	GAEYASFLQ	EAQVPFLSLE	RCSAPDVHGS
550	560	570	580	590	600
SILPGMLCAG	FLEGGTDACQ	GDSGGPLVCE	DQAAERRLTL	QGIISWGSGC	GDRNKPGVYT
610					
DVAYYLAWIR	EHTVS				

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Direct BLAST submission at
[EMBLnet-CH and CSCS](#)
(Switzerland)



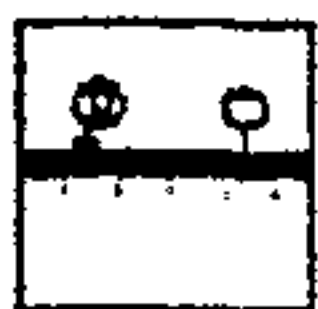
Direct BLAST submission at [NCBI](#)
(Bethesda, USA)



[ScanProsite](#), [ProfileScan](#)



Tools
Sequence analysis tools: [ProtParam](#),
[ProtScale](#), [Compute pI/Mw](#), [PeptideMass](#),
[Dotlet](#) (Java)



Feature table [viewer](#) (Java)



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<http://www.expasy.ch/cgi-bin/sprot-search-de>

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[\[General\]](#) [\[Name and origin\]](#) [\[References\]](#) [\[Comments\]](#) [\[Cross-references\]](#) [\[Keywords\]](#) [\[Features\]](#)
[\[Sequence\]](#) [\[Tools\]](#)

General information about the entry

Entry name HGFA_HUMAN
Primary accession number Q04756
Secondary accession number Q14726
Entered in SWISS-PROT in Release 29, June 1994
Sequence was last modified in Release 29, June 1994
Annotations were last modified in Release 40, October 2000

Name and origin of the protein

Protein name HEPATOCYTE GROWTH FACTOR ACTIVATOR
[Precursor]
Synonyms EC 3.4.21.-
HGF ACTIVATOR
HGFA
Gene name HGFAC
From Homo sapiens (Human) [TaxID: 9606]
Taxonomy Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.

References

- [1] SEQUENCE FROM NUCLEIC ACID, AND PARTIAL SEQUENCE.
TISSUE=Liver, and Serum;
MEDLINE=93252878; PubMed=7683665; [NCBI, ExPASy, EBI, Israel, Japan]
Miyazawa K., Shimomura T., Kitamura A., Kondo J., Morimoto Y., Kitamura N.;
"Molecular cloning and sequence analysis of the cDNA for a human serine protease responsible for
activation of hepatocyte growth factor. Structural similarity of the protease precursor to blood
coagulation factor XII.";
J. Biol. Chem. 268:10024-10028(1993).
- [2] SEQUENCE OF 40-655 FROM NUCLEIC ACID.
Zhao S., Odell C.;
Submitted (FEB-1996) to the EMBL/GenBank/DBJ databases.

Comments

- **FUNCTION:** ACTIVATES HEPATOCYTE GROWTH FACTOR (HGF) BY CONVERTING IT FROM A SINGLE CHAIN TO A HETERODIMERIC FORM.
- **SUBUNIT:** DIMER OF A SHORT CHAIN AND A LONG CHAIN LINKED BY A DISULFIDE BOND.
- **SUBCELLULAR LOCATION:** SECRETED AS AN INACTIVE SINGLE-CHAIN PRECURSOR AND IS THEN ACTIVATED TO A HETERODIMERIC FORM.
- **TISSUE SPECIFICITY:** LIVER.
- **SIMILARITY:** CONTAINS 2 EGF-LIKE DOMAINS.
- **SIMILARITY:** CONTAINS 1 FIBRONECTIN TYPE I DOMAIN.
- **SIMILARITY:** CONTAINS 1 FIBRONECTIN TYPE II DOMAIN.
- **SIMILARITY:** CONTAINS 1 KRINGLE DOMAIN.
- **SIMILARITY:** BELONGS TO PEPTIDASE FAMILY S1; ALSO KNOWN AS THE TRYPSIN FAMILY.
- **CAUTION:** IT IS UNCERTAIN WHETHER MET-1 IS THE INITIATOR.

Copyright

FIG. 12

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Cross-references

EMBL	D14012; BAA03113.1; -. [EMBL / GenBank / DDBJ] [CoDingSequence] Z69923; CAA93803.1; -. [EMBL / GenBank / DDBJ] [CoDingSequence]
PIR	A46688; A46688.
MIM	604552 [NCBI / EBI].
GeneCards	HGFAC .
HSSP	P00763 ; 1DPO. [HSSP ENTRY / PDB]
MEROPS	S01.228 ; -. IPR001314 : Chymotrypsin. IPR000561 : EGF-like. IPR000742 : EGF_2. IPR000562 : FN_Type_II. IPR000001 : Kringle. IPR001254 : Trypsin. IPR000083 : fibronectin type 1. Graphical view of domain structure . PF00008 : EGF; 2. PF00039 : fn1; 1. PF00040 : fn2; 1. PF00051 : kringle; 1. PF00089 : trypsin; 1. PR00013 : FNTYPEII. PR00018 : KRINGLE. PR00722 : CHYMOTRYPSIN. PD000995 : -; 1. [Domain structure / List of seq. sharing at least 1 domain]. SM00181 : EGF; 2. SM00058 : FN1; 1. SM00059 : FN2; 1. SM00130 : KR; 1. SM00020 : Tryp SPc; 1. PS00022 : EGF_1; 2. PS01186 : EGF_2; 1. PS01253 : FIBRONECTIN_1; 1. PS00023 : FIBRONECTIN_2; 1. PS00021 : KRINGLE_1; 1. PS50070 : KRINGLE_2; 1. PS50240 : TRYPSIN_DOM; 1. PS00134 : TRYPSIN_HIS; 1. PS00135 : TRYPSIN_SER; 1.
InterPro	
Pfam	
PRINTS	
ProDom	
SMART	
PROSITE	
BLOCKS	Q04756 .
DOMO	Q04756 .
ProtoMap	Q04756 .
PRESAGE	Q04756 .
DIP	Q04756 .
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Keywords

[Hydrolase](#); [Glycoprotein](#); [Plasma](#); [Serine protease](#); [Kringle](#); [Signal](#); [EGF-like domain](#); [Repeat](#); [Zymogen](#).

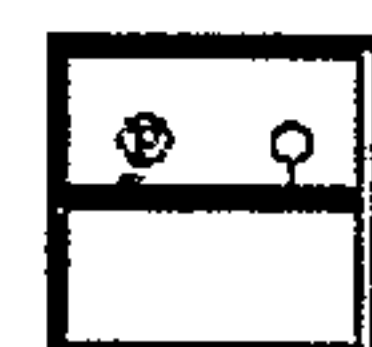
Features

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<http://www.expasy.ch/cgi-bin/sprot-search-de>

Key	From	To	Length	Description
SIGNAL	<u>1</u>	<u>30</u>	30	
PROPEP	<u>31</u>	<u>372</u>	342	CLEAVED IN ACTIVE FORM.
CHAIN	<u>373</u>	<u>407</u>	35	HEPATOCYTE GROWTH FACTOR ACTIVATOR SHORT CHAIN.
CHAIN	<u>408</u>	<u>655</u>	248	HEPATOCYTE GROWTH FACTOR ACTIVATOR LONG CHAIN.
DOMAIN	<u>108</u>	<u>148</u>	41	FIBRONECTIN TYPE-II.
DOMAIN	<u>160</u>	<u>198</u>	39	EGF-LIKE 1.
DOMAIN	<u>200</u>	<u>240</u>	41	FIBRONECTIN TYPE-I.
DOMAIN	<u>241</u>	<u>279</u>	39	EGF-LIKE 2.
DOMAIN	<u>286</u>	<u>367</u>	82	KRINGLE.
DOMAIN	<u>408</u>	<u>655</u>	248	SERINE PROTEASE.
ACT_SITE	<u>447</u>	<u>447</u>		CHARGE RELAY SYSTEM (BY SIMILARITY).
ACT_SITE	<u>497</u>	<u>497</u>		CHARGE RELAY SYSTEM (BY SIMILARITY).
ACT_SITE	<u>598</u>	<u>598</u>		CHARGE RELAY SYSTEM (BY SIMILARITY).
DISULFID	<u>108</u>	<u>133</u>		BY SIMILARITY.
DISULFID	<u>122</u>	<u>148</u>		BY SIMILARITY.
DISULFID	<u>164</u>	<u>175</u>		BY SIMILARITY.
DISULFID	<u>169</u>	<u>186</u>		BY SIMILARITY.
DISULFID	<u>188</u>	<u>197</u>		BY SIMILARITY.
DISULFID	<u>202</u>	<u>230</u>		BY SIMILARITY.
DISULFID	<u>228</u>	<u>237</u>		BY SIMILARITY.
DISULFID	<u>245</u>	<u>256</u>		BY SIMILARITY.
DISULFID	<u>250</u>	<u>267</u>		BY SIMILARITY.
DISULFID	<u>269</u>	<u>278</u>		BY SIMILARITY.
DISULFID	<u>286</u>	<u>367</u>		BY SIMILARITY.
DISULFID	<u>307</u>	<u>349</u>		BY SIMILARITY.
DISULFID	<u>338</u>	<u>362</u>		BY SIMILARITY.
DISULFID	<u>394</u>	<u>521</u>		INTRACHAIN (BY SIMILARITY).
DISULFID	<u>432</u>	<u>448</u>		BY SIMILARITY.
DISULFID	<u>440</u>	<u>510</u>		BY SIMILARITY.
DISULFID	<u>535</u>	<u>604</u>		BY SIMILARITY.
DISULFID	<u>567</u>	<u>583</u>		BY SIMILARITY.
DISULFID	<u>594</u>	<u>622</u>		BY SIMILARITY.
CARBOHYD	<u>48</u>	<u>48</u>		N-LINKED (GLCNAC...) (POTENTIAL).
CARBOHYD	<u>290</u>	<u>290</u>		N-LINKED (GLCNAC...) (POTENTIAL).
CARBOHYD	<u>468</u>	<u>468</u>		N-LINKED (GLCNAC...) (POTENTIAL).
CARBOHYD	<u>492</u>	<u>492</u>		N-LINKED (GLCNAC...) (POTENTIAL).
CARBOHYD	<u>546</u>	<u>546</u>		N-LINKED (GLCNAC...) (POTENTIAL).
CONFLICT	<u>644</u>	<u>644</u>		R -> Q (IN REF. 2).

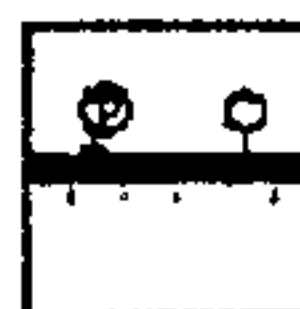
[Feature
aligner](#)[Feature table
viewer](#)**Sequence information**Length: 655 AA [This is the
length of the unprocessed
precursor]Molecular weight: 70681 Da
[This is the MW of the
unprocessed precursor]CRC64: 2CF72F1E1B862ED7 [This is
a checksum on the sequence]

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```
      10      20      30      40      50      60
      |      |      |      |      |      |
MGRWAWVPSP WPPPGLGPFL LLLLLLLLLP RGFQPPQGGN RTESPEPNAT ATPAIPILV
      70      80      90     100     110     120
      |      |      |      |      |      |
TSVTSETPAT SAPEAEGPOS GGLPPPPRAV PSSSSPQAQA LTEDGRPCR FFRYGGRLH
     130     140     150     160     170     180
      |      |      |      |      |      |
ACTSEGAHR KWCATTHNYD RDRAWGYCVE ATPPPGGPAA LDPCASGPCL NGGSCSNTQD
     190     200     210     220     230     240
      |      |      |      |      |      |
PQSYHCSCPR AFTGKDCGTE KCFDETRYEY LEGGDRWARV RQGHVEQCEC FGGRTWCEGT
     250     260     270     280     290     300
      |      |      |      |      |      |
RHTACLSSPC LGGGTCHLIV ATGTTVCACP PGFAGRLCNI EPDERCFLGN GTGYRGVAST
     310     320     330     340     350     360
      |      |      |      |      |      |
SASGLSCLAW NSDLLYQELH VDSVGAAALL GLGPHAYCRN PDNDERPWCY VVKDSALSWE
     370     380     390     400     410     420
      |      |      |      |      |      |
YCRLEACESL TRVQLSPDLL ATLPEPASPG RQACGRRHKK RTFLRPRIIG GSSSLPGSHP
     430     440     450     460     470     480
      |      |      |      |      |      |
WLAAIYIGDS FCAGSLVHTC WVVSAAHCFS HSPPRDSVSV VLGQHFFNRT TDVTQTFGIE
     490     500     510     520     530     540
      |      |      |      |      |      |
KYIPYTLYSV FNPSDHDVLV IRLKKKGDR ATRSQFVQPI CLPEPGSTFP AGHKCQIAGW
     550     560     570     580     590     600
      |      |      |      |      |      |
GHLDENVSGY SSSLREALVP LVADHKCSSP EVYGADISPN MLCAGYFDCK SDACQGDSGG
     610     620     630     640     650
      |      |      |      |      |
PLACEKNGVA YLYGIISWGD GCGRLHKPGV YTRVANYVDW INDRIRPPRR LVAPS
```

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[EMBnet-CH and CSCS](#)
(Switzerland)Direct BLAST submission at [NCBI](#)
(Bethesda, USA)[ScanProsite](#), [ProfileScan](#)Tools Sequence analysis tools: [ProtParam](#),
[ProtScale](#), [Compute pI/Mw](#), [PeptideMass](#),
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PIR Entry Request

http://pir.georgetown.edu/cgi-bin/pirsearch



PIR Entry JC4795



Home	About PIR	FTP Site	PIR-PSD	iProClass	Database Info	Database Retrieval	Sequence Search	Site Map
----------------------	---------------------------	--------------------------	-------------------------	---------------------------	-------------------------------	------------------------------------	---------------------------------	--------------------------

Search:

in the PIR1 section of the Protein Sequence Database, release 68.03, 24-May-2001, assembled and annotated by the PIR-International.
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[Submission Form](#) create a [BLAST \(at PIR\)](#) ☒ submission form for JC4795

[Annotation](#) || [Sequence](#) || [Composition Table](#) || Download: ([CODATA](#) || [FASTA](#) || [XML](#))

```

ENTRY          JC4795  #type complete
TITLE          plasma hyaluronan-binding protein precursor - human
ALTERNATE_NAMES  hepatocyte growth factor activator-like protein; PHBP
CONTAINS       serine proteinase (EC 3.4.21.-)
ORGANISM       #formal_name Homo sapiens #common_name man
               #cross-references taxon:9606
DATE           15-Oct-1995 #sequence_revision 16-Aug-1996 #text_change
               16-Jul-1999
ACCESSIONS     JC4795
REFERENCE      JC4795
               #authors Choi-Miura, N.H.; Tobe, T.; Sumiya, J.; Nakano, Y.; Sano,
               Y.; Mazda, T.; Tomita, M.
               #journal J. Biochem. (1996) 119:1157-1165
               #title Purification and characterization of a novel
               hyaluronan-binding protein (PHBP) from human plasma: It
               has three EGF, a kringle and a serine protease domain,
               similar to hepatocyte growth factor activator.
               #cross-references MUID:96425001
               #accession JC4795
               ##molecule_type mRNA
               ##residues 1-560 ##label CHO
               ##cross-references GB:S83182; NID:g1836158; PIDN:AAB46909.1;
               PID:g1836159
               ##experimental_source plasma
               ##note parts of this sequence, including the amino ends of the
               mature chains, were determined by protein sequencing
GENETICS
               #gene GDB:HABP2; HABP; PHBP; HGFAL
               ##cross-references GDB:4573962
COMPLEX        a disulfide-bonded heterodimer of chains produced from the
               same precursor; the catalytic chain is degraded to a 17K
               chain lacking the active site serine residue
CLASSIFICATION #superfamily plasma hyaluronan-binding protein; EGF
               homology; kringle homology; trypsin homology
KEYWORDS       chondroitin sulfate proteoglycan; glycoprotein; growth
               factor; hyaluronic acid; hydrolase; kringle; plasma;
               serine proteinase
FEATURE
               1-23 #domain signal sequence #status predicted #label
               SIG\
               24-313 #product plasma hyaluronan-binding protein, 50K
               chain #status predicted #label 50K\
               77-108 #domain EGF homology #label EG1\
               115-147 #domain EGF homology #label EG2\
               154-187 #domain EGF homology #label EG3\
               194-276 #domain kringle homology #label KRI\
               314-550 #domain trypsin homology #label TRY\
               314-516 #product plasma hyaluronan-binding protein,
               catalytic chain #status predicted #label CAT\
               54,207 #binding site carbohydrate (Asn) (covalent)
               #status predicted\
               77-88,82-97,99-108,
               115-125,120-136,
               138-147,154-165,
               159-176,178-187,
               194-276,215-257,
               246-271,301-435,
               347-363,355-424,

```

FIG. 13

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PIR Entry Request

<http://pir.georgetown.edu/cgi-bin/pirsearch>

447-515,477-493,
505-533 #disulfide bonds #status predicted\
362,405,509 #active site His, Asp, Ser #status predicted
SUMMARY #length 560 #molecular_weight 62671

SEQUENCE

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31 E S L D P D W T P D Q Y D Y S Y E D Y N Q E E N T S S T L T
61 H A E N P D W Y Y T E D Q A D P C Q P N P C E H G G D C L V
91 H G S T F T C S C L A P F S G N K C Q K V Q N T C K D N P C
121 G R G Q C L I T Q S P P Y Y R C V C K H P Y T G P S C S Q V
151 V P V C R P N P C Q N G A T C S R H K R R S K F T C A C P D
181 Q F K G K F C E I G S D D C Y V G D G Y S Y R G K M N R T V
211 N Q H A C L Y W N S H L L L Q E N Y N M F M E D A E T H G I
241 G E H N F C R N P D A D E K P W C F I K V T N D K V K W E Y
271 C D V S A C S A Q D V A Y P E E S P T E P S T K L P G F D S
301 C G K T E I A E R K I K R I Y G G F K S T A G K H P W Q A S
331 L Q S S L P L T I S M P Q G H F C G G A L I H P C W V L T A
361 A H C T D I K T R H L K V V L G D Q D L K K E E F H E Q S F
391 R V E K I F K Y S H Y N E R D E I P H N D I A L L K L K P V
421 D G H C A L E S K Y V K T V C L P D G S F P S G S E C H I S
451 G W G V T E T G K G S R Q L L D A K V K L I A N T L C N S R
481 Q L Y D H M I D D S M I C A G N L Q K P G Q D T C Q G D S G
511 G P L T C E K D G T Y Y V Y G I V S W G L E C G K R P G V Y
541 T Q V T K F L N W I K A T I K S E S G F
```

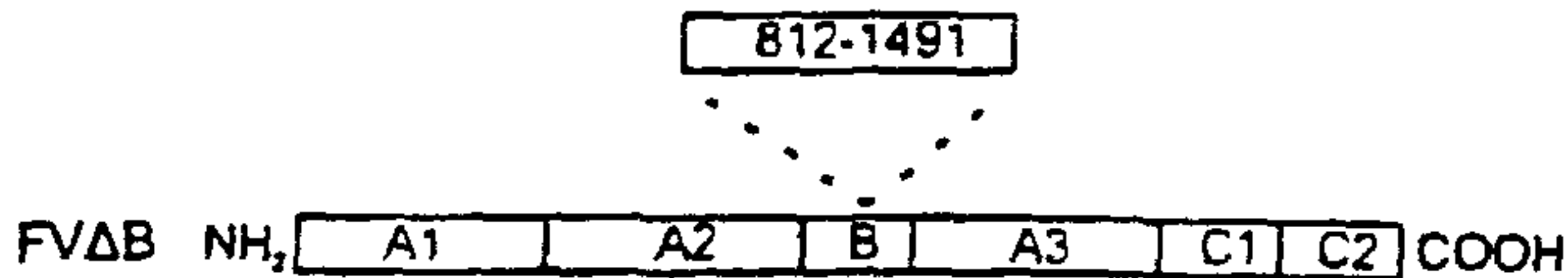
Enzyme Links for JC4795:

EC-IUBMB: EC [3.4.21.-](#)KEGG: EC [3.4.21.-](#)BRENDA: EC [3.4.21.-](#)WIT: EC [3.4.21.-](#)MetaCyc: EC [3.4.21.-](#)

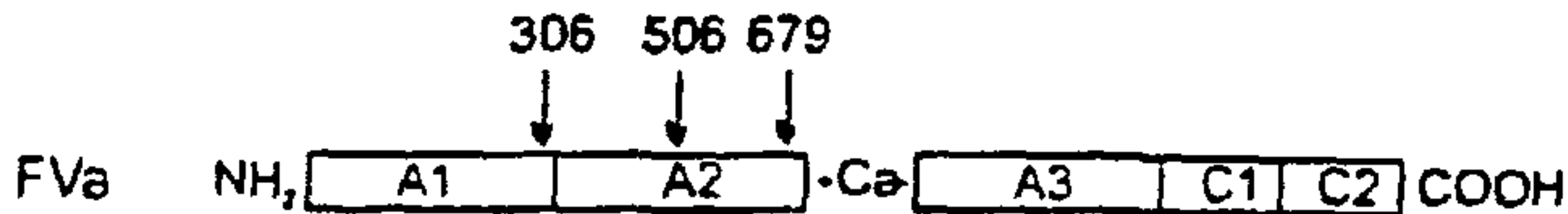
Associated Alignments:

[DA0704](#) EGF homology - Type A[DA0705](#) EGF homology - Type B[DA1052](#) kringle homology - selected coagulation factors[DA1082](#) trypsin homologyLink to *iProClass* (Superfamily classification and Alignment):[iProClass](#) Report for JC4795 at PIR.

A



B



C

