Title: THERAPEUTIC FRAGMENTS OF VON WILLEBRAND FACTOR

Abstract

A polypeptide patterned upon a parent polypeptide and comprising the amino acid sequence of that fragment of mature von Willebrand factor subunit which begins approximately at residue 441 (arginine) and ends at approximately residue 733 (valine), or any subset thereof, in which one or more of the cysteine residues normally present in the parent polypeptide, or subset thereof, have been deleted and/or replaced by one or more other amino acids, said polypeptide having therefore less tendency than the parent polypeptide, or subset thereof, to form intra or interchain disulfide bonds in aqueous media at a physiological pH, and including also a DNA sequence encoding an aforementioned polypeptide; and also a biologically functional expression plasmid or viral expression vector containing DNA encoding for an aforementioned polypeptide and capable of being replicated in a host cell; a therapeutic composition comprising one or more of the involved polypeptides; and also a method for inhibiting thrombosis in a patient which comprises administering to such patient an effective amount of the therapeutic composition.
| AT  | Austria     | ES  | Spain   | MG  | Madagascar |
| AU  | Australia   | FI  | Finland | ML  | Mali       |
| BB  | Barbados    | FR  | France  | MN  | Mongolia   |
| BE  | Belgium     | GA  | Gabon   | MR  | Mauritania |
| BF  | Burkina Faso| GB  | United Kingdom | MW | Malawi    |
| BG  | Bulgaria    | GN  | Guinea  | NL  | Netherlands |
| BJ  | Benin       | GR  | Greece  | NO  | Norway     |
| BR  | Brazil      | HU  | Hungary | PL  | Poland     |
| CA  | Canada      | IT  | Italy   | RO  | Romania    |
| CF  | Central African Republic | JP | Japan   | SD  | Sudan      |
| CG  | Congo       | KP  | Democratic People's Republic | SE | Sweden     |
| CH  | Switzerland | KR  | Republic of Korea | SN | Senegal    |
| CI  | Côte d'Ivoire| LI  | Liechtenstein | SU* | Soviet Union |
| CM  | Cameroon    | LK  | Sri Lanka | TD  | Chad       |
| CS  | Czechoslovakia| LL | Luxembourg | TG  | Togo       |
| DE# | Germany     | MC  | Monaco  | US  | United States of America |
| DK  | Denmark     |     |         |     |            |

Any designation of "SU" has effect in the Russian Federation. It is not yet known whether any such designation has effect in other States of the former Soviet Union.
THERAPEUTIC FRAGMENTS OF VON WILLEBRAND FACTOR

Field of the Invention

This invention relates to polypeptides which are useful in the treatment of vascular disorders such as thrombosis. This invention relates also to polypeptides which are useful in the treatment of hemorrhagic diseases, such as von Willebrand disease (vWD). This invention further relates to the production by recombinant DNA-directed methods of pharmacologically useful quantities of the polypeptides of the present invention.

The term "hemostasis" refers to those processes which comprise the defense mechanisms of the body against loss of circulating blood caused by vascular injury. Processes which are normal as a physiologic response to vascular injury may lead in pathologic circumstances, such as in a patient afflicted with atherosclerotic vascular disease or chronic congestive heart failure, to the formation of undesired thrombi (clots) with resultant vascular occlusion. Impairment of blood flow to organs under such circumstances may lead to severe pathologic states, including myocardial
infarction, a leading cause of mortality in developed countries.

The restriction or termination of the flow of blood within the circulatory system in response to a wound or as a result of a vascular disease state involves a complex series of reactions which can be divided into two processes, primary and secondary hemostasis. Primary hemostasis refers to the process of platelet plug or soft clot formation. The platelets are non-nucleated discoid structures approximately 2-5 microns in diameter derived from megakaryocytic cells. Effective primary hemostasis is accomplished by platelet adhesion, the interaction of platelets with the surface of damaged vascular endothelium on which are exposed underlying collagen fibers and/or other adhesive macromolecules such as proteoglycans and glycosaminoglycans to which platelets bind.

Secondary hemostasis involves the reinforcement or crosslinking of the soft platelet clot. This secondary process is initiated by proteins circulating in the plasma (coagulation factors) which are activated during primary hemostasis, either in response to a wound or a vascular disease state. The activation of these factors results ultimately in the production of a polymeric matrix of the protein fibrinogen (then called fibrin) which reinforces the soft clot.

The present invention relates to antiplatelet drugs. Antiplatelet drugs include drugs which suppress primary hemostasis by altering platelets or their interaction with other circulatory system components.
Specific antiplatelet drugs operate by one or several mechanisms. A first example involves reducing the availability of ionized calcium within the platelet cytoplasm thereby impairing activation of the platelet and resultant aggregation. Pharmaceuticals representative of this strategy include prostacyclin, and also Persantine® (dipyridamole) which may affect calcium concentrations by affecting the concentration of cyclic AMP. Numerous side effects related to the administration of these compounds have been reported. An additional class of antiplatelet drugs acts by inhibiting the synthesis of thromboxane A₂ within the platelet, reducing the platelet activation response. Non-steroidal anti-inflammatory agents, such as ibuprofen, phenolbutazone and naphthoxane may produce a similar effect by competitive inhibition of a particular cyclooxygenase enzyme, which catalyzes the synthesis of a precursor of thromboxane A₂. A similar therapeutic effect may be derived through the administration of aspirin which has been demonstrated to irreversibly acetylate a cyclooxygenase enzyme necessary to generate thromboxane A₂. A third anti-platelet mechanism has involved the platelet membrane so as to interfere with surface receptor function. One such drug is dextran, a large branched polysaccharide, which is believed to impair the interaction of fibrinogen with platelet receptors that are exposed during aggregation. Dextran is contraindicated for patients with a history of renal problems or with cardiac impairment. The therapeutic ticlopidine is stated to inhibit platelet adhesion and aggregation by suppressing the binding of von Willebrand factor and/or
fibrinogen to their respective receptors on the platelet surface. However, it has been found that ticlopidine possesses insufficient specificity to eliminate the necessity of administering large doses which, in turn, may be associated with clinical side effects.

The aforementioned pharmaceuticals are foreign to the body and may cause numerous adverse clinical side effects, there being no way to prevent such compounds from participating in other aspects of a patient's physiology or biochemistry, particularly if high doses are required. It would be desirable to provide for pharmaceuticals having such specificity for certain of the reactions of hemostasis, that they could be administered to patients at low doses, such doses being much less likely to produce adverse effects in patients.

An example of a pharmaceutical which is representative of a therapeutic that is derived from natural components of the hemostatic process is described in EPO Publication No. 317278. This publication discloses a method for inhibiting thrombosis in a patient by administering to the patient a therapeutic polypeptide comprised of the aminoterminus region of the α chain of platelet membrane glycoprotein Ib, or a subfragment thereof.

The present invention is directed to the provision of antithrombotic polypeptides derived from von Willebrand factor, one of the proteins of the hemostatic mechanism.
Summary of the Present Invention

In accordance with the present invention, there is provided a polypeptide patterned upon a parent polypeptide and comprising the amino acid sequence of that fragment of mature von Willebrand factor subunit which beings approximately at residue 441 (arginine) and ends at approximately residue 733 (valine), or any subset thereof, in which one or more of the cysteine residues normally present in the parent polypeptide, or subset thereof, have been deleted and/or replaced by one or more other amino acids, said polypeptide having therefore less tendency than the parent polypeptide, or subset thereof, to form intra or interchain disulfide bonds in aqueous media at a physiological pH.

The polypeptides of the invention are expressed in both recombinant bacterial and recombinant eucaryotic host cells.

Modification in accordance with the present invention of a parent polypeptide by deleting or replacing one or more cysteine residues normally present in the parent polypeptide, or subset thereof, results in a polypeptide having less tendency than the parent polypeptide, or subset thereof, to form intrachain or interchain disulfide bonds in aqueous media at physiological pH. The practical effect of this is that the polypeptide of the present invention exhibits a higher degree of therapeutic activity than the parent polypeptide and improved stability and solubility. For convenience, a polypeptide of the present invention is often referred to herein as being "mutant".
In preferred form, it is recommended that the invention be practiced by substituting for one or more cysteine residues particular amino acid residues which are expected to not significantly alter the predetermined tertiary structure of the parent cysteine-containing VWF polypeptide or fragment thereof. This contributes to maintaining the therapeutic potency of the mutant polypeptide. Preferred amino acid replacements include glycine, serine, alanine, threonine and asparagine, with serine, alanine and glycine being preferred.

Another aspect of the invention is based upon the discovery that cysteine residues 509 and 695 of the mature von Willebrand factor subunit normally form an intrachain disulfide bond which confers upon the subunit, or a fragment thereof, a particular tertiary structure which is involved in the binding of von Willebrand factor, or of a therapeutically useful polypeptide derived therefrom, to the glycoprotein Ib receptor of platelets. Accordingly, another aspect of the invention comprises a polypeptide comprising the amino acid sequence from approximately residue 441 (arginine) to approximately residue 733 (valine) of mature von Willebrand factor subunit, or any subset of said sequence which contains residues 509 (cysteine) and 695 (cysteine), wherein one or more of cysteine residues 459, 462, 464, 471, and 474 are deleted or replaced by one or more other amino acids. A preferred polypeptide is one in which each of cysteine residues 459, 462, 464, 471 and 474 is replaced by a glycine residue and in which cysteine residues 509 and 695 are linked by an intrachain disulfide bond.
Another aspect of the present invention is the provision of a therapeutic composition which comprises a therapeutically effective amount of a polypeptide of the present invention and a pharmaceutically acceptable carrier therefor.

Still another aspect of the invention provides a method of inhibiting thrombosis in a patient which comprises administering to the patient an effective amount of one or more of the therapeutic compositions of the invention. It is expected that therapeutic compositions comprising one or more of the polypeptides of this invention will be substantially less toxic or cause fewer adverse physiological effects in patients than currently available antiplatelet drugs such as dipyridamole.

A preferred method for generating the polypeptides of the present invention is to subject a DNA nucleotide sequence coding for the von Willebrand factor subunit, or fragments thereof, to mutagenesis resulting in the deletion of cysteine residues, or their replacement by other amino acid species. The resultant encoding DNA may be inserted into recombinant bacterial host cells for expression of the vWF polypeptide.

The invention provides also for eucaryotic host cells containing recombinant vWF DNA sequences from which are expressed therapeutically-active polypeptides related to the 52/48 kDa tryptic fragment or domain of vWF. The polypeptides are successfully secreted from the host cells.
The polypeptides expressed in this way have certain advantageous features when compared with polypeptides expressed from recombinant bacterial host cells.

1) The polypeptides of the present invention assume three dimensional structures which are characteristic of the domain which exists in mature circulating von Willebrand factor and they have properly formed disulfide bonds.

2) The polypeptides of the present invention are closer analogs of the natural vWF 52/48 functional domain in that they have the glycosylation characteristic of said domain.

Such polypeptides, when present in monomeric form, may be used as antithrombotic agents. In dimerized form (which dimerization further validates that the polypeptides have natural structural domains), they can be used as antihemorrhagic agents. The therapeutic properties of polypeptides of the present invention can be enhanced by altering the glycosylation thereof, as described in detail hereinbelow.

Of importance to the proper three-dimensional folding and secretion of the polypeptides of the invention is the initial attachment there to of a signal peptide sequence which is also effective in causing secretion of other polypeptides unrelated to vWF from the same or other host cells.

In accordance with the practice of this invention, there are provided therapeutically useful polypeptides which are effective in preventing adhesion of platelets
to surfaces, in inhibiting activation or aggregation of platelets, and in inhibiting thrombosis. More specifically there are provided glycosylated polypeptides which are effective in inhibiting the binding of von Willebrand factor multimers to platelets and which are created by expression in mammalian cells of mutant human von Willebrand factor subunit DNA sequences. Such polypeptides show less tendency than homologous non-mutant polypeptides to form interchain disulfide bonds which tend to adversely affect the therapeutic utility thereof.

Accordingly, there is provided a polypeptide patterned upon a parent polypeptide and comprising the amino acid sequence of that fragment of mature von Willebrand factor subunit which begins approximately at residue 441 (arginine) and ends at approximately residue 733 (valine), or any subset thereof, wherein one or more of cysteine residues 459, 462 and 464 are deleted and/or replaced by one or more other amino acids, and wherein said polypeptide has less tendency than said parent polypeptide to form interchain disulfide bonds.

It is believed that this aspect of the invention will be most commonly practiced by substituting for one or more of the specified cysteine residues particular amino acid residues which do not significantly alter the predetermined tertiary structure of the parent cysteine-containing VWF polypeptide, or of a fragment thereof, thereby maintaining the therapeutic potency of the mutant polypeptide. Suitable amino acid replacements include glycine, serine, alanine,
threonine or asparagine with alanine and glycine being most preferred.

The present invention is concerned also with the preparation by recombinant DNA-directed methods of a monomeric and properly glycosylated fragment of von Willebrand factor subunit which is useful in inhibiting thrombosis in a patient. The recombinant methods minimize the production of structures which tend to adversely affect the desired therapeutic activity of the desired monomeric form of the fragment, for example, dimers, multimers, or aggregates of said fragment. Accordingly there is provided a process for producing from DNA corresponding to that fragment of mature von Willebrand factor subunit comprising essentially the amino acid sequence from approximately residue 441 (arginine) to approximately residue 730 (asparagine), a biologically active monomer of said subunit fragment having an apparent molecular weight by SDS-polyacrylamide gel electrophoresis of approximately 52 kDa which process comprises the steps of:

(A) constructing a DNA sequence encoding the subunit fragment which contains upstream from the fragment encoding region thereof, and in proper reading frame therefor, a signal peptide sequence;

(B) mutagenizing the DNA sequence to reduce the number of cysteine codons capable of specifying cysteine residues normally involved in interchain disulfide contacts;

(C) inserting the DNA sequence into a suitable vector to create a construct comprising an expression plasmid or viral expression vector, said construct being capable of
directing the expression in and secretion from eucaryotic cells of said monomeric subunit fragment;

(D) transforming a eucaryotic host cell with said construct; and

(E) culturing said transformed host cell under conditions which cause expression within and secretion from said host cell of the monomeric subunit fragment, said conditions also permitting glycosylation of said fragment.

The present invention is also concerned with the preparation of polypeptides which are useful in the treatment of hemorrhagic disease such as von Willebrand disease (vWD). Specifically, the present invention is concerned with preparation by recombinant DNA-directed methods of particular fragments of von Willebrand factor which fragments are capable of performing a bridging function between the GPIb(α) receptor of the platelet membrane and a similar receptor on another platelet cell, or between such a receptor and components of the subendothelium including collagen, thereby performing the crucial physiological role of native multimeric von Willebrand factor in affected individuals. Accordingly there is provided a process for producing from DNA corresponding to that monomeric fragment of mature von Willebrand factor subunit comprising essentially the amino acid sequence from approximately residue 441 (arginine) to approximately residue 730 (asparagine), or a subfragment thereof containing one or more of residue positions 459, 462, and 464, a biologically active dimer of said monomeric
fragment or subfragment which process comprises the steps of:

(A) constructing a DNA sequence encoding the monomeric fragment or subfragment which further contains upstream from the fragment encoding region thereof and in proper reading frame therefor, a signal peptide sequence;

(B) inserting the DNA sequence into a suitable vector to create a construct comprising an expression plasmid or viral expression vector, said construct being capable of directing the expression in, and secretion from, eucaryotic cells of said monomeric fragment or subfragment;

(C) transforming a eucaryotic host cell with said construct;

(D) culturing said transformed host cell under conditions that cause expression within the host cell and secretion therefrom of the dimeric form of the monomeric fragment or subfragment and under which the monomeric fragment or subfragment assumes a tertiary structure suitable for dimerization and the dimerization thereof, and under which there is effected glycosylation of said monomeric subunit fragment or subfragment or of a dimeric form thereof.

Another aspect of the invention is based upon the discovery that the ristocetin-induced interaction between cloned 116 kDa vWF fragment and platelets can be enhanced by reducing the amount of glycosylation on the 116 kDa fragment. This discovery is useful in the design of additional polypeptides effective in the
treatment of thrombosis or of von Willebrand disease. Accordingly, there is provided a mutant polypeptide patterned upon a parent polypeptide which comprises the amino acid sequence of that fragment of mature von Willebrand factor subunit which begins approximately at residue 449 (valine) and ends at approximately residue 728 (lysine), or a dimer thereof, from which parent one or more serine, threonine or asparagine residues which are sites of O- or N-linked glycosylation have been deleted or replaced by one or more other amino acids, said mutant polypeptide having less glycosylation when said mutant polypeptide is expressed from recombinant DNA in a host eucaryotic cell than the species of the parent polypeptide having an apparent molecular weight of 52 kDa, as measured by SDS-polyacrylamide gel electrophoresis.

It is believed the invention, and the mutagenesis and protein expression procedures thereof, will be widely practiced in the art to generate mutant mature von Willebrand factor subunit fragments with improved solubility, stability and therapeutic activity.

Although the invention is described initially in connection with the expression and secretion from mammalian cells of certain glycosylated fragments of mature von Willebrand factor having therapeutic utility, it should be understood that it is applicable also to the expression in mammalian cells of other therapeutic polypeptides in which secretion from said cells of said polypeptides is facilitated by an additional sequence of amino acids which are also encoded by a DNA for the therapeutic polypeptide and which comprise human von Willebrand factor signal
peptide, or a subset thereof, and the amino terminal region of the von Willebrand factor propeptide. Accordingly, there is also provided a polypeptide which is capable of directing the transport of additional polypeptide sequence across the membrane of the endoplasmic reticulum of a cell and which is comprised of a domain (A) and a domain (B) as follows:

- **Domain (A)**: any subset of the signal peptide of human von Willebrand factor subunit which signal peptide is capable of being recognized by the endoplasmic reticulum and/or by translocation receptors which complex with the endoplasmic reticulum and/or the signal peptide; and

- **Domain (B)**: a peptide sequence consisting essentially of up to the first ten residues of the amino terminal end of von Willebrand factor propeptide; said domain (B) being connected by amide linkage to the carboxy terminus of domain (A) and capable of being connected by amide linkage to the amino terminus of said additional polypeptide sequence; which polypeptide comprising domain (A) and domain (B) contains a sufficient subset of the sequence of the human von Willebrand factor signal peptide and propeptide to facilitate cleavage in a manner such that there remains attached to the amino terminal end of the additional polypeptide sequence a subset of the sequence derived from domains (A) and (B), and wherein therapeutic
activity of the additional polypeptide sequence is retained in whole or part.

Speaking more generally, there is also provided a process for producing from DNA a therapeutic polypeptide comprising:

(A) constructing a DNA sequence encoding the therapeutic polypeptide which contains upstream from the polypeptide encoding region thereof, and in proper reading frame therefor, a DNA sequence which itself corresponds to a signal peptide and directly downstream therefrom a semipolar or polar spacer sequence;

(B) inserting the resultant DNA sequence into a suitable vector to create a construct comprising an expression plasmid or viral expression vector which is capable of directing the expression in and secretion from eucaryotic host cells of said therapeutic polypeptide;

(C) transforming a eucaryotic host cell with said construct; and

(D) culturing said transformed host cell under conditions which cause expression within and secretion from said host cell of the therapeutic polypeptide.

**Brief Description of the Drawings**

Figure 1 is a table which shows the previously reported amino acid and DNA sequence for the mature von Willebrand factor subunit (human) between residue 431 and residue 750.
Figure 2 is a graph which shows the inhibition of botrocetin-induced binding of vWF to platelets by a cysteine-free mutant polypeptide of the present invention.

Figure 3 is a graph which shows the inhibition of the binding of an anti GP1b monoclonal antibody to platelet by a mutant polypeptide of the present invention.

Figure 4 is a map of pCDM8 plasmid.

Definitions

Unless indicated otherwise herein, the following terms have the indicated meanings.

Coding Sequence (Encoding DNA) - DNA sequences which, in the appropriate reading frame, code for the amino acids of a protein. For the purpose of the present invention, it should be understood that the synthesis or use of a coding sequence may necessarily involve synthesis or use of the corresponding complementary strand, as shown by: 5'–CGG·GGA·GGA–3'/3'–GCC·CCT·CCT–5' which "encodes" the tripeptide NH₂-arg-gly-gly-CO₂H. A discussion of or claim to one strand is deemed to refer to or to claim the other strand and the double stranded counterpart thereof as is appropriate, useful or necessary in the practice of the art.

cDNA - A DNA molecule or sequence which has been enzymatically synthesized from the sequence(s) present in an mRNA template.
Transcribed Strand - The DNA strand whose nucleotide sequence is read 3' → 5' by RNA polymerase to produce mRNA. This strand is also referred to as the noncoding strand.

Coding Strand or Non-Transcribed Strand - This strand is the antiparallel compliment of the transcribed strand and has a base sequence identical to that of the mRNA produced from the transcribed strand except that thymine bases are present (instead of uracil bases of the mRNA). It is referred to as "coding" because like mRNA, and when examined 5' → 3', the codons for translation may be directly discerned.

Biological Activity - One or more functions, effects of, activities performed or caused by a molecule in a biological context (that is, in an organism or in an in vitro facsimile). A characteristic biological activity of the 116 kDa homodimeric fragment of the mature von Willebrand factor subunit is the potential ability to bind to more than one platelet GPIb receptor thereby enabling the molecule to facilitate aggregation of platelets in the presence of ristocetin. Other resultant or related effects of the 116 kDa species include function as a thrombotic and the induction of platelet activation, and/or adhesion to surfaces.

Similarly, a characteristic biological activity of the 52/48 kDa monomeric fragment of the mature von Willebrand factor subunit is the potential ability to bind to only one platelet GPIb receptor thereby enabling the molecule to inhibit botrocetin-induced binding of multimeric vWF to platelets. Other resultant or related effects of the undimerized 52/48 kDa species include inhibition of platelet activation,
aggregation, or adhesion to surfaces, and the inhibition of thrombosis.

Reducing Conditions - Refers to the presence of a "reducing" agent in a solution containing von Willebrand factor, or polypeptides derived therefrom, which agent causes the disruption of disulfide bonds of the vWF. However, consistent with usage typical in the art, the "reducing" agent such as dithiothreitol (DTT) causes a vWF disulfide bond to be broken by forming a disulfide bond between a vWF cysteine and the DTT with no net change in oxidation state of the involved sulfur atoms.

Promoter - DNA sequences upstream from a gene which promote its transcription.

Cloning Vehicle (Vector) - A plasmid, phage DNA or other DNA sequence which is able to replicate in a host cell, typically characterized by one or a small number of endonuclease recognition sites at which such DNA sequences may be cut in a determinable fashion for the insertion of heterologous DNA without attendant loss of an essential biological function of the DNA, e.g., replication, production of coat proteins or loss of expression control regions such as promoters or binding sites, and which may contain a selectable gene marker suitable for use in the identification of host cells transformed therewith, e.g., tetracycline resistance or ampicillin resistance.

Plasmid - A nonchromosomal double-stranded DNA sequence comprising an intact "replicon" such that the plasmid is replicated in a host cell. When the plasmid is
placed within a procaryotic or eucaeryotic host cell, 
the characteristics of that cell may be changed (or 
transformed) as a result of the DNA of the plasmid. 
For example, a plasmid carrying the gene for 
tetracycline resistance (Tet^R) transforms a cell 
previously sensitive to tetracycline into one which is 
resistant to it. A cell transformed by a plasmid is 
called a "transformant."

Expression Plasmid - A plasmid into which has been 
inserted the DNA being cloned, such as the von 
Willebrand factor structural gene. The DNA sequence 
inserted therein may also contain sequences which 
control the translation of mRNA resultant therefrom, 
and contain restriction endonuclease sites which 
facilitated assembly of, and may facilitate further 
modification of, said expression plasmid. An 
expression plasmid is capable of directing, in a host 
cell, the expression therein of the encoded polypeptide 
and usually contains a transcription promoter upstream 
from the DNA sequence of the encoded structural gene. 
An expression plasmid may or may not become integrated 
into the host chromosomal DNA. For the purpose of this 
invention, an integrated plasmid is nonetheless 
referred to as an expression plasmid.

Viral Expression Vector - A viral expression vector is 
similar to an expression plasmid except that the DNA 
may be packaged into a viral particle that can 
transfect cells through a natural biological process.

Downstream - A nucleotide of the transcribed strand of 
a structural gene is said to be downstream from another 
section of the gene if the nucleotide is normally read
by RNA polymerase after the earlier section of the gene. The complimentary nucleotide of the nontranscribed strand, or the corresponding base pair within the double stranded form of the DNA, are also denominated downstream.

Additionally, and making reference to the direction of transcription and of translation within the structural gene, a restriction endonuclease sequence added upstream (or 5') to the gene means it is added before the sequence encoding the amino terminal end of the protein, while a modification created downstream (or 3') to the structural gene means that it is beyond the carboxy terminus-encoding region thereof.

von Willebrand factor (vWF) - It is understood that all references herein to von Willebrand factor refer to vWF in humans. The term "von Willebrand factor" is intended to include within its scope any and all of the terms which are defined directly below.

Additionally, von Willebrand factor is found as a component of the subendothelial matrix, as a component of the α-granules secreted by activated platelets, and as a circulating blood plasma protein. It is possible that the three-dimensional subunit structure or multisubunit structure of vWF varies in these different contexts potentially caused, for example, by differences in glycosylation. Such differences do not prevent useful therapeutic vWF-derived polypeptides from being produced from the vWF DNA sequences of endothelial cells or megakaryocytes according to the practice of this invention.
Furthermore it is possible that there are minor biologically unimportant differences between the actual DNAs and polypeptides manipulated or otherwise utilized in the practice of the invention and the structural sequences of amino acids or nucleotides thereof as reported herein. It is understood that the invention encompasses any such biologically unimportant variations.

Pre-pro-vWF - von Willebrand factor is subject to extensive posttranslational processing. "Pre-pro-vWF" contains (from the N to the C terminus) a signal peptide comprised of approximately 22 amino acid residues, a propeptide of approximately 741 amino acids, and then the approximate 2,050 residues of circulating vWF.

Pro-vWF - The signal peptide has been removed from pre-pro-vWF.

Mature vWF - Circulating vWF as found in the plasma or as bound to the subendothelium. It consists of a population of polypeptide monomers which are typically associated into numerous species of multimers thereof, each subunit of which being 2,050 residues in length. Additionally, when expressed in mammalian cells, mature vWF is usually glycosylated.

Signal Peptide (Sequence) - A signal peptide is the sequence of amino acids in a newly translated polypeptide which signals translocation of the polypeptide across the membrane of the endoplasmic reticulum and into the secretory pathway of the cell. A signal peptide typically occurs at the beginning
(amino terminus) of the protein and is 20-40 amino acids long with a stretch of approximately 5-15 hydrophobic amino acids in its center. Typically the signal sequence is proteolytically cleaved from the protein during, or soon after, the process of translocation into the endoplasmic reticulum. That portion of a gene or cDNA encoding a signal peptide may also be referred to as a signal sequence.

Table 1 shows the standard three letter designations for amino acids as used in the application.

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Detailed Description of the Invention

As set forth above, both the antithrombotic and antihemorrhagic polypeptides of the present invention are based upon fragments of the natural occurring
Protein von Willebrand factor (hereinafter "vWF"). For background purposes, there is set forth hereafter information concerning this protein and its role in hemostasis and thrombosis.

Description of the Role of vWF in Hemostasis and Thrombosis

vWF performs an essential role in normal hemostasis during vascular injury and is also of central importance in the pathogenesis of acute thrombotic occlusions in diseased blood vessels. Both of these roles involve the interaction of vWF with platelets which are induced to bind at the affected site and are then crosslinked. It is believed that single platelets first adhere to a thrombogenic surface after which they become activated, a process involving major metabolic changes and significant morphological changes within the platelet. Activation is evidenced by the discharge of platelet storage granules containing adhesive substances such as von Willebrand factor (an adhesive protein), and the expression on the surface of the platelet of additional functional adhesive sites. Once activated, and as a part of normal hemostasis, platelet cells become aggregated, a process which involves extensive crosslinking of the platelet cells with additional types of adhesive proteins.

As stated above, these processes are normal as a physiologic response to vascular injury. However, they may lead in pathologic circumstances, such as in diseased vessels, to formation of undesired platelet thrombi with resultant vascular occlusion.
Other circumstances in which it is desirable to prevent deposition of platelets in blood vessels include the prevention and treatment of stroke, and to prevent occlusion of arterial grafts. Platelet thrombus formation during surgical procedures may also interfere with attempts to relieve preexisting vessel obstructions.

The adhesion of platelets to damaged or diseased vessels occurs through mechanisms that involve specific platelet membrane receptors which interact with specialized adhesive molecules. One such platelet receptor is the glycoprotein Ib-IX complex which consists of a noncovalent association of two integral membrane proteins, glycoprotein Ib (GPIb) and glycoprotein IX (GPIX). The adhesive ligand of the GPIb-IX complex is the protein von Willebrand factor which is found as a component of the subendothelial matrix, as a component of the α-granules secreted by activated platelets, and also as a circulating blood plasma protein. The actual binding site of the vWF to the GPIb-IX receptor has been localized on the amino terminal region of the α chain of glycoprotein Ib which is represented by GPIb(α).

It is believed that the interaction of multimeric vWF with glycoprotein Ib-IX complex (at GPIb(α)) results in platelet activation and facilitates the recruitment of additional platelets to a growing thrombus. The rapidly accumulating platelets are also crosslinked (aggregated) by the binding of fibrinogen at platelet glycoprotein IIb-IIIa receptor sites, and possibly also by vWF at these sites, and/or at additional glycoprotein Ib-IX receptor sites. In
addition, the glycoprotein IIb/IIIa receptor may also be involved in the formation of the initial monolayer of platelets. Of particular importance in this process is the multimeric and multivalent character of circulating vWF, which enables the macromolecule to effectively carry out its binding and bridging functions.

Inactivation of the GPIbα or GPIIb/IIIa receptors on the platelets of a patient or inactivation of the binding sites for vWF located in the subendothelium of a patient’s vascular system, thereby inhibiting the bridging ability of vWF, would be of great medical importance for treating or inhibiting thrombosis. Accordingly, the present invention relates to the development of polypeptides which are effective in accomplishing the foregoing.

Although preventing unwanted thrombi is of great importance, there are circumstances where promoting thrombus formation is desirable. von Willebrand disease, the most common of the bleeding disorders, is the term used to describe a heterogeneous disease state which results when von Willebrand factor is produced in inadequate quantities or when circulating vWF molecules are somehow defective. Various subtypes of the disease have been described. It is apparent that supplying the bridging function of vWF is of central importance in the treatment of patients afflicted with von Willebrand disease. The present invention is concerned also with preparation of fragments of von Willebrand factor capable of performing a bridging function between the GPIb(α) receptor or GPIIb/IIIa receptor of the platelet membrane and a receptor on another platelet, or between
such a receptor and components of the subendothelium, thereby performing in affected individuals the crucial physiological role of native multimeric von Willebrand factor.

5 Information Concerning the Structure of vWF and the Design of Therapeutics Derived Therefrom

The domain of the von Willebrand factor subunit which binds to the platelet membrane glycoprotein Ib-IX receptor (GPIb(a)) has been identified within a fragment of vWF. The fragment may be generated by trypsin digestion, followed by disulfide reduction, and extends from approximately residue 449 (valine) of the circulating subunit to approximately residue 728 (lysine) thereof. Current evidence indicates that this segment also contains (between residues 509 and 695 thereof) binding domains for components of the subendothelium, such as collagen and proteoglycans, although other regions of the mature vWF subunit may be more important in recognizing these substances (an additional proteoglycan or heparin binding site is located in residues 1-272 of the mature subunit and an additional collagen binding site within residues 910-1110 thereof).

Figure 1 (SEQ ID NO: 1) shows the previously reported amino acid and DNA sequence for the mature von Willebrand factor subunit (human) between residue 431 and residue 750. The 52/48 kDa fragment produced by tryptic digestion has an amino terminus at residue 449 (valine) and extends approximately to residue 728 (lysine). Amino acids are shown by standard three letter designations. The DNA sequence is represented by the coding strand (non-transcribed strand). Very
little polymorphism has been reported in the 52/48 human sequence with one significant exception - histidine/aspartic acid at position 709, see Mancuso, D.J. et al. J. Biol. Chem., 264(33), 19514-19527, Table V, (1989). DNA sequences used for the experiments described in the Example section below contain an aspartic acid codon for residue 709 (codon GAC), although placement of histidine at residue position 709 (the other known naturally occurring amino acid at this position in the human sequence, codon CAC) is also useful in the practice of the invention.

With respect to the therapeutic antithrombotic polypeptides of the present invention, the following information concerning vWF is of particular interest.

A fragment of mature von Willebrand factor having platelet glycoprotein Ib(α) binding activity and of approximately 116,000 (116 kDa) molecular weight is isolated by digesting vWF with trypsin. If the 116 kDa fragment is treated with a reducing agent capable of cleaving disulfide bonds, a pair of identical fragments is generated. Each of the identical fragments (which together comprise the 116 kDa polypeptide) has an apparent molecular weight of about 52,000 (52 kDa). (Polypeptide molecular weight are typically measured by migration, relative to standards, in a denaturing gel electrophoresis system. Weight values which result are only approximate.)

Typically, the 52,000 molecular weight fragment is referred to as a "52/48" fragment reflecting the fact that human enzyme systems glycosylate the fragment contributing to its molecular weight. The amount of
glycosylation varies from molecule to molecule, with two weights, 52,000 and 48,000, being most common.

The 52/48 fragment has been demonstrated to have as its amino-terminus residue 449 (valine) of the mature subunit, and as its carboxy-terminus residue 728 (lysine) thereof. Without the additional weight contributed by glycosylation, the polypeptide has a molecular weight of approximately 38,000.

The 52/48 fragment has been demonstrated to competitively inhibit the binding of von Willebrand factor to platelets. However, manipulation of the 52/48 fragment or its unglycosylated 38 kDa equivalent has proved difficult. Successful manipulation of the fragment has typically required that the cysteine residues thereof be reduced and permanently alkylated. Without this treatment, undesired reaction of the cysteine residues thereof invariably occurs, leading to the formation of insoluble and biologically inactive polypeptide aggregates unsuited for effective use as therapeutics.

It is known that the residue 449-728 fragment of mature von Willebrand factor subunit, which contains the platelet glycoprotein Ib(α) binding domain, has cysteine residues at positions 459, 462, 464, 471, 474, 509 and 695. It is known also that all of the cysteine residues of the mature vWF subunit are involved in disulfide bonds. (Legaz, et al., *J. Biol. Chem.*, 248, 3946-3955 (1973)).

471 and 474 as being involved in an intrachain
disulfide bond. Residues 509 and 695 were identified
as being involved in a disulfide bond, although it was
not demonstrated whether this pairing was intrachain or
interchain (that is, within the same mature vWF
subunit).

Mohri, H. et al. J. Biol. Chem., 263(34), 17901-
17904 (1988) inhibited the ristocetin-induced binding
of $^{125}$I-labelled multimeric vWF to formalin-fixed
platelets with peptide subfragments of the 449-728
subunit fragment. Peptide subfragments fifteen
residues in length were synthesized and tested. Those
peptides which represent subunit sequence contained
within, or overlapping with, two distinct regions,
Leu$^{69}$ to Asp$^{68}$ and Glu$^{69}$ to Val$^{713}$ were found to be
active.

Mohri concluded that the GPIb(α) binding domain of
vWF was formed by residues contained in two
discontinuous sequences Cys$^{474}$-Pro$^{488}$ and Leu$^{594}$-Pro$^{708}$
maintained in proper conformation in native vWF by
disulfide bonding, although the authors were unable to
identify the cysteine residue which formed the
stabilizing bond(s) and whether the bonds were intra or
interchain.

The present invention provides for polypeptides
derived from the residue 449-728 region of the mature
von Willebrand factor subunit which are useful in the
treatment of vascular disorders such as thrombosis.

Such molecules can be made most efficiently from
DNA which encodes that fragment of mature von
Willebrand factor subunit comprising essentially the amino acid sequence from approximately residue 441 (arginine) to approximately residue 733 (valine), or which encodes any subset of said amino acid sequence, or a mutant polypeptide fragment, or subset thereof, which contains fewer cysteine residues than that of the comparable wild-type amino acid sequence. A preferred method for the preparation of the molecules comprises culturing a host organism transformed with a biologically functional expression plasmid which contains a mutant DNA sequence encoding a portion of said von Willebrand factor subunit under conditions which effect expression of the mutant von Willebrand factor fragment, or a subset thereof, by the host organism and recovering said fragment therefrom.

A preferred means for effecting mutagenesis of cysteine codons in a vWF DNA to codons encoding amino acids incapable of disulfide bonding is based upon the site directed mutagenesis procedure of Kunkel, T.A., Proc. Natl. Acad. Sci. U.S.A., 82, 488-492 (1985). Such mutant DNA sequences may then be expressed from either recombinant-bacterial or recombinant-eucaryotic host cell systems.

First Embodiment of the Invention

An important aspect of this embodiment of the invention is the provision of compositions of said vWF-derived polypeptides which are less prone to aggregation and denaturation caused by undesired disulfide bonding within the inclusion bodies of host expression cells (or resultant from inclusion body solubilization procedures) than previous preparations.
The development employs mutagenesis to limit the number of cysteine residues present within said polypeptides.

**Mutagenesis of vWF DNA Encoding The Mature Subunit Residue 449-728 Region**

A variety of molecular biological techniques are available which can be used to change cysteine codons for those of other amino acids. Suitable techniques include mutagenesis using a polymerase chain reaction, gapped-duplex mutagenesis, and differential hybridization of an oligonucleotide to DNA molecules differing at a single nucleotide position. For a review of suitable codon altering techniques, see Kraik, C. "Use of Oligonucleotides for Site Specific Mutagenesis", *Biotechniques*, Jan/Feb 1985 at page 12.

In the practice of this embodiment, it preferred to use the site-directed or site-specific mutagenesis procedure of Kunkel, T.A., *Proc. Natl. Acad. Sci. USA*, 82, 488-492 (1985). This procedure takes advantage of a series of steps which first produces, and then selects against, a uracil-containing DNA template. Example 1 of the present invention explains in detail the mutagenesis techniques used to create mutant vWF cDNA.

Other publications which disclose site-directed mutagenesis procedures are: Giese, N.A. et al., *Science*, 236, 1315 (1987); U.S. Patent No. 4,518,584; and U.S. Patent No. 4,959,314.

It is also preferred in the practice of this embodiment to cause to be substituted for one or more of the cysteine codons of the wild type DNA sequence codons for one or more of the following amino acids:
alanine, threonine, serine, glycine, and asparagine. Replacement with alanine and glycine codons is most preferred. The selection of a replacement for any particular codon is generally independent of the selection of a suitable replacement at any other position.

The following are representative examples of the types of codon substitutions which can be made, using as an example cysteine residue 459:

(A) the codon for cysteine 459 could be replaced by a codon for glycine; or

(B) the codon for cysteine 459 could be replaced by two or more codons such as one for serine and one for glycine, such replacement resulting in a new amino acid sequence: -His^{458}-Ser^{459(6)}-Gly^{459(6)}-Gln^{460}-; or

(C) the codon for cysteine 459 could be deleted from the cDNA, such deletion resulting in a shortened amino acid sequence represented by: -His^{458}-Gln^{460}-; or

(D) one or more codons for residues adjacent to cysteine residue 459 could be deleted along with codon 459 as represented by: -Glu^{457}-Gln^{460}-. 

It is contemplated that codons for amino acids other than alanine, threonine, serine, glycine or asparagine will also be useful in the practice of the invention depending on the particular primary, secondary, tertiary and quaternary environment of the target cysteine residue.
It is considered desirable in the practice of this embodiment to provide as a replacement for any particular cysteine residue of the 449-728 tryptic vWF subunit fragment an amino acid which can be accommodated at the cysteine position with minimal perturbation of the secondary structure (such as α-helical or β-sheet) of the wild type amino acid sequence subsegment within which the cysteine position is located. In the practice of the present invention, alanine, threonine, serine, glycine and asparagine will generally be satisfactory because they are, like cysteine, neutrally charged and have side chains which are small or relatively small in size.

Substantial research has been conducted on the subject of predicting within which types of structural domains of proteins (α-helix, β-sheet, or random coil) one is most likely to find particular species of amino acids. Serine is a preferred amino acid for use in the practice of this invention because it most closely approximates the size and polarity of cysteine and is believed not to disrupt α-helical and β-sheet domains.

Reference, for example, to Chou, P.Y. et al., *Biochemistry*, 13(2), 211-222 (1974) and Chou, P.Y. et al., "Prediction of Protein Conformation,"

*Biochemistry*, 13(2), 222-244 (1974) provides further information useful in the selection of replacement amino acids. Chou, P.Y. et al. predicted the secondary structure of specified polypeptide sequence segments based on rules for determining which species of amino acids therein are likely to be found in the center of, for example, an alpha helical region, and which residues thereof would be likely to terminate
propagation of a helical zone, thus becoming a boundary residues or helix breakers. According to Chou, P.Y. et al., supra, at 223, cysteine and the group of threonine, serine, and asparagine are found to be indifferent to $\alpha$-helical structure, as opposed to being breakers or formers of such regions. Thus, threonine, serine and asparagine are likely to leave unperturbed an $\alpha$-helical region in which a potential target cysteine might be located. Similarly, glycine, alanine and serine were found to be more or less indifferent to the formation of $\beta$-regions. It is noted that serine, threonine and asparagine residues represent possible new sites of glycosylation making them potentially unsuitable replacement residues at certain positions in secretory proteins subject to glycosylation.

Generally, the primary consideration which should be taken into account in connection with selecting suitable amino acid replacements is whether the contemplated substitution will have an adverse effect on the tertiary structure of the fragment. Thus, other amino acids may be suitable as acceptable substitutes for particular cysteine residues as long as the new residues do not introduce undesired changes in the tertiary structure of the 449-728 fragment. Reactivity with NMC-4 antibody is recommended as a test of whether a mutant polypeptide has the desired therapeutic properties.

Particularly preferred mutant polypeptides of the present invention are patterned upon a monomeric form of the residue 449-728 domain of the mature subunit fragment, as opposed to a dimer thereof which could provide a bridging function between two platelets.
Normally, those codons in a vWF DNA fragment for specific cysteines which normally participate in interchain disulfide bonding should be replaced. Cysteine codons encoding residues which form intrachain disulfide bonds should be left unmutated, if the intrachain bond is demonstrated to confer upon the subunit fragment important structural features, and if conditions can be found which allow the intrachain bond to form properly.

More specifically, preparation of a mutant polypeptide fragment which corresponds to that fragment of mature von Willebrand subunit having an amino terminus at residue 441 (arginine) and a carboxy terminus at residue 733 (valine), but which differs therefrom in that each of the cysteine residues thereof is replaced by a glycine residue is disclosed.

The embodiment also teaches that retention of a certain disulfide bond within polypeptides corresponding to the 449-728 vWF subunit region is particularly important for the design of therapeutic molecules derived therefrom. In this regard there is provided a mutant vWF fragment expressed by p5E plasmids, as described in Example 4, and containing an intrachain disulfide bond.

Important factors involved in the design of preferred mutant polypeptides of the invention are described hereafter.

Potential binding sites for collagens and heparin-like glycosaminoglycans exist in the 449-728 tryptic fragment in the loop region between cysteine residues.
509 and 695. In the event that binding at these sites impairs the antithrombotic therapeutic utility of the molecule by, for example, also providing bridging to collagen, the polypeptide can be redesigned (for example, by chemical synthesis or proteolysis) to delete the loop region.

von Willebrand factor polypeptides derived from bacterial expression systems substantially lack the glycosylation vWF normally acquires as a result of post-translational processing such as in the Golgi apparatus and Weibel-Palade bodies. The present invention includes within its scope molecules which are made by E.coli BL21(DE3) or other suitable procaryotic host cells and which are enzymatically or chemically glycosylated to more resemble the molecules expressed by mammalian cells.

Alternatively, the DNA encoding sequences can be transferred to expression plasmids or viral expression vectors capable of causing expression in mammalian host cells to provide normal glycosylation.

It has been established that both platelets and von Willebrand factor molecules contain large numbers of negative charges such as, for example, those contributed by sialic acid. Such charges can facilitate desirable mutual repulsion of the molecules under non-injury conditions. The addition of one or more positively charged residues of lysine and/or of arginine extending from the amino and/or from the carboxy terminus of the 52/48 tryptic fragment or recombinant equivalents thereof can overcome electrical repulsions with respect to the GPIb-IX receptor
facilitating use of the fragment as an antithrombotic therapeutic.

In addition, and with respect to polypeptides patterned upon the 449-728 vWF subunit fragment, it is within the scope of the invention to remove certain cysteine residues by site directed mutagenesis and thereafter inactivating any remaining cysteine residues by chemical inactivation thereof, such as, for example, by S-carboxymethylation.

A mutant polypeptide that is insoluble can be made soluble by covalently linking to it a subdomain of a water soluble polymer, for example, a polyacrylamide. Other techniques can also be used to impart solubility to an otherwise insoluble polypeptide.

In light of the aforementioned, which is generally applicable to all the polypeptides of the invention, there follows hereafter a discussion of means by which mutant polypeptides of the first embodiment of the invention can be prepared.

To accomplish this, a cDNA clone encoding the von Willebrand factor gene (for the pre-propeptide) was utilized. The cDNA was then subjected to enzymatic amplification in a polymerase chain reaction using oligonucleotides which flanked the indicated region. The first oligonucleotide representing coding strand DNA contained an EcoRI site 5' to the codon for residue 441 (arginine) and extended to the codon for residue 446 (glycine). The second oligonucleotide, corresponding to non-coding strand DNA, encoded amino acids 725 to 733 and encoded 3' to codon 733 a HindIII
restriction sequence. The resultant double stranded von Willebrand factor cDNA corresponding to the amino acid sequence from residue 441 to residue 733 (of the mature subunit) was then inserted, using EcoRI and HindIII restriction enzymes, into the double stranded replicative form of bacteriophage M13mp18 which contains a multiple cloning site having compatible EcoRI and HindIII sequences. Following the procedure of Kunkel, T.A., *Proc. Natl. Acad. Sci. USA*, 82, 488-492 (1985), site directed mutagenesis was performed using hybridizing oligonucleotides suitable for replacing all of the cysteine codons (residue positions 459, 462, 464, 471, 474, 509 and 695) with individual glycine codons (see Example 1) or, for example, 5 of the cysteine codons, residue positions 459, 462, 464, 471 and 474, with individual glycine codons (see Example 4). Mutant double stranded vWF cDNA fragments derived from the procedure were removed from M13mp18 phage by treatment with EcoRI and HindIII restriction endonucleases, after which the ends of the vWF cDNA fragments were modified with BamHI linkers.

The two types of mutant vWF cDNA, containing either 5 or 7 Cys to Gly mutations, were then separately cloned into the pET-3A expression vector (see Rosenberg, A.H. et al., *Gene*, 56, 125-136 (1987)) for expression from *E.coli* strain BL21(DE3), Novagen Co., Madison, WI. pET-3A vehicle containing cDNA for the vWF subunit fragment with 7 cysteine-to-glycine mutations is referred to as "p7E", and as "p5E" when the contained vWF cDNA fragment encoded the 5 above specified cysteine-to-glycine mutations. Mutant von Willebrand factor polypeptides produced by bacterial cultures containing expression plasmid p5E were
compared with those expressed from cultures containing p7E plasmids. The p5E molecule is capable of forming a disulfide bond between cysteine residue 509 and 695 whereas the p7E molecule cannot.

The mutant polypeptides were not secreted by the bacterial host cells, but rather accumulated in poorly soluble aggregates ("inclusion bodies") from which the polypeptides were successfully solubilized following the procedure of Example 1 (p7E) and Example 4 (p5E).

Polypeptides expressed from p7E and p5E plasmids were characterized by SDS-polyacrylamide gel electrophoresis and immunoblotting (Examples 2 and 5). Under reducing conditions both plasmids express polypeptide species having an apparent molecular weight of approximately 38,000 as measured by SDS-polyacrylamide gel electrophoresis, as would be predicted from the unglycosylated molecular weight of the expected amino acid sequences.

The behavior of p5E and p7E extracts was examined using immunological methods (see Example 5). vWF-specific murine monoclonal antibodies RG-46 and NMC-4 were used as probes. RG-46 has been demonstrated to recognize as its epitope a linear sequence of amino acids, comprising residues 694 to 708 within the mature von Willebrand factor subunit. The binding of this antibody to its determinant is essentially conformation independent. Mohri, H. et al., J. Biol. Chem., 263(34), 17901-17904 (1988).

NMC-4 however, has as its epitope the domain of the von Willebrand factor subunit which contains the glycoprotein Ib binding activity. Mapping of the
epitope has demonstrated that it is contained within two discontinuous domains (comprising approximately mature vWF subunit residues 474 to 488 and also approximately residues 694 to 708) brought into disulfide-dependent association, Mohri, H. et al., supra, although it could not be determined whether the disulfide bond conferring this tertiary conformation in the native vWF molecule was intrachain or interchain. Id. at 17903.

Accordingly, 7.5 µg samples (of protein) were first run on 10% SDS-polyacrylamide gels so that the antigenic behavior of particular bands (under reducing and nonreducing conditions) could be compared with results obtained by Coomassie blue staining. Immunoblotting ("Western Blotting") according to a standard procedure, Burnette, A. Anal. Biochem., 112, 195-203 (1981), was then performed to compare p5E and p7E extracts.

It has been determined that, under nonreducing conditions, the single chain p5E polypeptide fragment (representing the sequence from residue 441 to residue 733) displays an approximate 120 fold increase in binding affinity for NMC-4 compared to the comparable cysteine-free species isolated from p7E. After electrophoresis under reducing conditions (utilizing 100 mM DTT), the single chain p5E species shows a remarkably decreased affinity for NMC-4, which was then very similar to that of the cysteine-free p7E species under either reduced or nonreduced conditions. NMC-4 also failed, under reducing or non-reducing conditions, to recognize as an epitope disulfide-linked dimers from the p5E extract.
The nitrocellulose filters used to produce autoradiographs based on NMC-4 were rescreened with RG-46 by subtracting the initial NMC-4 exposure response, which was kept low through a combination of low antibody titer and short exposure time. The binding of RG-46 to the 36,000 kDa p7E polypeptide on the filters was the same whether reducing or non-reducing conditions were chosen, consistent with the replacement of all cysteines by glycine in the expressed polypeptide.

A large molecular weight vWF antigen (reactive to RG-46) was present in the p5E polypeptide extract under nonreducing conditions. These p5E vWF aggregates (reflecting interchain disulfide bonds) migrated under reducing conditions in the same position as the p7E polypeptide indicating disruption of their disulfide contacts. However, the large p5E interchain disulfide aggregates which are readily recognized under nonreducing conditions by RG-46 were not recognized by NMC-4 under either reducing or nonreducing conditions. It was thus demonstrated that the disulfide bond between residues 509 and 695 in native multimeric vWF subunits represents an intrachain contact.

The disulfide bond between residues 471 and 474 of the mature vWF subunit has previously been shown to be an intrachain contact, thus the aforementioned embodiment is able to suggest that interchain disulfide bond(s) in multimeric mature vWF would be formed using one or more of cysteine residues 459, 462 or 464.

A wide variety of expression plasmids or viral expression vectors are suitable for the expression of
the 441-733 fragment, or similar vWF fragments. Representative examples include pBR322, and derivatives thereof such as pET-1 through pET-7. Suitable host cells include the bacterial genera of *Escherichia* and *Bacillus*. Of importance in the selection of an expression system is the recommended presence of a high efficiency transcription promoter directly adjacent to the vWF cloned DNA insert. Mutant vWF cDNA fragments may also be cloned in eucaryotic host cells.

This discovery is expected to be particularly useful in the design of therapeutic vWF polypeptides patterned upon the 52/48 tryptic fragment (for use as antithrombotics) or patterned instead upon the 116 kDa homodimer thereof (for use as antihemorrhagics).

**Second Embodiment of the Invention**

Many of the factors described above with respect to the design of and expression of therapeutic fragments of vWF from recombinant bacterial cells are applicable to the design of and expression of vWF fragments from eucaryotic host cells. Such applicability is readily apparent to those skilled in the art.

This second embodiment includes within its scope the recognition of certain of the roles performed by cysteine residues present in the residue 449-728 primary sequence fragment of the mature vWF subunit. In this connection, this embodiment confirms that the cysteine 509-695 disulfide bond is an intrachain bond and provides for effective therapeutics incorporating the 509-695 bond for the purpose of treating
thrombosis, or for the purpose of treating von Willebrand's disease.

Both the antithrombotic polypeptides and antihemorrhagic polypeptides of this the second embodiment of the invention are based upon that amino acid sequence domain which comprises approximately residues 449 to 728 of the mature von Willebrand factor subunit and which, if fully glycosylated, would be equivalent in weight to the 52/48 kDa vWF subunit fragment. In practice it is difficult to derive therapeutically useful quantities of such polypeptides from blood plasma. Difficulties include effective separation of 116 kDa and 52/48 kDa fragments from other components of tryptic digests and effective sterilization of blood-derived components from human viruses such as hepatitis and AIDS. In addition, methods reported in the literature to generate the 52/48 kDa monomer from the 116 kDa dimer have utilized complete disulfide reduction with resultant loss of tertiary structure. Certain important manipulations of the 52/48 fragment, such as replacement of selective cysteine residues to improve product utility and stability, can only be accomplished in a practical sense by recombinant DNA technology.

However, the production by recombinant DNA-directed means of therapeutic vWF polypeptides analogous to the 52/48 tryptic fragment has met with certain limitations. It is desirable that the polypeptide not only be made by the host cells but that it be correctly folded for maximum therapeutic utility. It is believed that the principal factor which has to date prevented the expression of the most
therapeutically active forms of the 52/48 fragment is the incorrect folding of the molecule caused by the linking up of cysteine residues to form incorrect disulfide contacts. In addition, such polypeptides appear to exhibit hydrophobic properties or solubility problems which would not be encountered if they were to be contained within the entirety of the natural vWF subunit, or were properly glycosylated.

Of critical importance, therefore, to the synthesis of vWF-derived therapeutic polypeptides is the selection of conditions which minimize the formation of improper disulfide contacts. Prior expression of such polypeptides from recombinant DNA in host bacterial cells has certain disadvantages. With reference to the first embodiment, newly produced vWF polypeptides are unable to escape from the host cells, causing them to be accumulated within insoluble aggregates therein (inclusion bodies) where the effective concentration of cysteine residues was extremely high. Under these circumstances, disulfide bonds not characteristic of the vWF molecule as it naturally exists in the plasma are encouraged to, and do, form either within the inclusion bodies or during attempts to solubilize the polypeptide therefrom.

This embodiment provides a solution to these difficulties by causing the vWF-derived polypeptides to be expressed in mammalian cells using a DNA sequence which encodes the polypeptide and which also encodes for a signal peptide, the presence of which causes the vWF polypeptide to be secreted from the host cells. Incorrect disulfide bond formation is minimized by
limiting the accumulation of high local concentrations of the polypeptide as in inclusion bodies.

In addition, enzymes present in the host eucaryotic cells, unlike bacteria, are able to glycosylate (add carbohydrate chains to) the vWF-derived polypeptides resulting in therapeutic molecules which more closely resemble domains of vWF molecules derived from human plasma.

The recombinant 116 kDa polypeptide generated according to this embodiment, without mutation of any of the cysteine codons therefor, is demonstrated to represent a dimer of the subunit fragment consisting of residues 441-730 and possesses an amount of glycosylation equivalent to that found in the comparable region of plasma-derived vWF.

There follows hereafter a description of the types of therapeutic vWF-derived polypeptides which have or may be generated according to the effective recombinant procedures of the second embodiment.

Recombinant vWF Polypeptides of the Second Embodiment

Stated broadly, this second embodiment includes any fragment of mature von Willebrand subunit comprising that sequence of amino acids between approximately residue 449 and approximately residue 728, or a subfragment thereof, from which at least one of cysteine residues 459, 462 and 464 thereof is removed. Such removal reduces the tendency of the fragment to form undesired interchain disulfide bonds
(and resultant dimers) with the result that therapeutic utility as an antithrombotic is improved.

A further aspect of the embodiment encompasses a glycosylated form of the above defined polypeptides.

In the design of antithrombotic polypeptides derived from the aforementioned region of vWF, it is preferred that cysteine residues be retained at positions 509 and 695 so that the tertiary structure of the GPIb(α) binding domain of the mature vWF subunit fragment is preserved.

Also preferred in the practice of the embodiment is a glycosylated polypeptide derived from the aforementioned region of vWF in which cysteine residues are retained at positions 509 and 695 and in which each of cysteine residues 459, 462 and 464 is deleted or replaced by residues of other amino acids.

Additionally preferred in the practice of the embodiment is a glycosylated polypeptide derived from the aforementioned region of vWF in which cysteine residues are retained at positions 509 and 695 and in which any one of cysteine residues 459, 462 and 464 is deleted or replaced by a single residue of another amino acid.

Important factors involved in the design of, or further modification to, the preferred mutant polypeptides (antithrombotics) of the invention are described hereafter.
Potential binding sites for collagens and glycosaminoglycans (or proteoglycans) exist in the 449-728 tryptic fragment in the loop region between cysteine residues 509 and 695. In the event that binding at these sites by such macromolecules impairs the antithrombotic therapeutic utility of any of the recombinant polypeptides of the invention by, for example, also providing bridging to collagen, the polypeptide can be redesigned (for example, by proteolysis, covalent labelling or mutagenesis) to delete or alter the loop region, or a subdomain thereof.

The second embodiment is also concerned with the preparation of polypeptides which are useful in the treatment of hemorrhagic disease. Stated broadly, there is provided a process for the production by recombinant DNA-directed methods of a dimeric polypeptide substantially equivalent to the 116 kDa tryptic fragment derived from circulating vWF. In accordance with the process, the monomeric fragment initially formed assumes a tertiary structure suitable for dimerization, and dimerization thereof is effected (see Example 7). In addition, the process conditions are such that it is possible to form a properly glycosylated dimeric polypeptide.

There follows hereafter a discussion of means by which polypeptides of the second embodiment can be prepared and, in particular, by which such polypeptides can be effectively secreted from host cells in proper folded form and possessing preferably only those disulfide bonds whose presence is consistent with therapeutic utility.
Preparation of Mutant Polypeptides of the Second Embodiment - Construction of Suitable DNA Sequences and Expression Plasmids

Essential elements necessary for the practice of the embodiment are: (A) a DNA sequence which encodes the residue 449-728 domain of the mature vWF subunit, or encodes a subdomain thereof; (B) an expression plasmid or viral expression vector capable of directing in a eucaryotic cell the expression therein of the aforementioned residue 449-728 domain, or subdomain thereof; and (C) a eucaryotic host cell in which said expression may be effected.

The expression of the DNA sequence of the von Willebrand factor subunit fragment is facilitated by placing a eucaryotic consensus translation initiation sequence and a methionine initiation codon upstream (5') to the residue 449-728 encoding DNA. The vWF DNA sequence may be a cDNA sequence, or a genomic sequence such as, for example, may be produced by enzymatic amplification from a genomic clone in a polymerase chain reaction. Expression of the residue 449-728 encoding sequence is further facilitated by placing downstream therefrom a translation termination codon such as TGA. The vWF-polypeptide so expressed typically remains within the host cells because of the lack of attachment to the nascent vWF polypeptide of a signal peptide. In such a situation, purification of proteins expressed therein and the extraction of pharmacologically useful quantities thereof are more difficult to accomplish than if the polypeptide were secreted into the culture medium of the host cells. Such expression systems are nonetheless useful for diagnostic assay purposes such as, for example, testing
the proper function of platelet GPIb-IX receptor complexes in a patient.

In the preferred practice of the invention in which the polypeptide is secreted from the host cell, there is provided a vWF-encoding DNA sequence for insertion into a suitable host cell in which there is also inserted upstream from the residue 449-728 encoding sequence thereof a DNA sequence encoding the vWF signal peptide (see Example 7). Other vWF-encoding DNA sequences corresponding to different regions of the mature vWF subunit, or corresponding to the propeptide, or to combinations of any of such regions, may be similarly expressed by similarly placing them downstream from a vWF signal peptide sequence in a suitable encoding DNA. When attached to the amino terminal end of the residue 449-728 fragment of the vWF subunit, the signal peptide causes the fragment to be recognized by cellular structures as a polypeptide of the kind to be processed for ultimate secretion from the cell, with concomitant cleavage of the signal polypeptide from the 449-728 fragment.

With respect to the construction of a eucaryotic expression system and the expression therein of the tryptic 52/48 kDa domain of mature subunit vWF (the residue 449-728 fragment), it has been found (see Example 7) to be convenient to manipulate a slightly larger fragment represented by residues 441 (arginine) to 730 (asparagine). Other similar fragments containing small regions of additional amino acids (besides the 449-728 residue sequence), which additional amino acids do not significantly affect the function of said fragment, may also be expressed.
Similarly, functional fragments may be expressed from which, when compared to the 449-728 fragment, several residues adjacent to the amino and carboxy terminals have been removed as long as the GPIb(α) binding sequences are not compromised.

It has also been found to be effective, with respect to the construction of a suitable DNA sequence for encoding and expressing the residue 441-730 fragment, to cause to be inserted between the DNA encoding the carboxy terminus of the signal peptide and the codon for residue 441, codons for the first three amino acids of the vWF propeptide (alanine-glutamic acid-glycine) said codons being naturally found directly downstream (3’) to the signal sequence in the human vWF gene. As is further elaborated below (see Example 17), the presence of such a propeptide sequence (a spacer) facilitates recognition by signal peptidase of a proper cleavage site which process generates a therapeutic vWF polypeptide of a proper size and facilitates secretion from the host cell of the therapeutic product. As elaborated below, this spacer sequence should be of semipolar or polar character.

In accordance with this invention, there is provided a spacer sequence comprising between one and up to the first ten residues of the amino terminal region of the vWF propeptide. It is within the scope of the invention to utilize longer propeptide encoding sequences with the understanding that the desired tertiary structure of the 441-730 residue sequence is not adversely affected.
A wide variety of expression plasmids or viral expression vectors are suitable for the expression of the residue 441-730 mature vWF subunit fragment or similar vWF fragments. One factor of importance in selecting an expression system is the provision in the plasmid or vector of a high efficiency transcription promoter which is directly adjacent to the cloned vWF insert.

Another factor of importance in the selection of an expression plasmid or viral expression vector is the provision in the plasmid or vector of an antibiotic resistance gene marker so that, for example, continuous selection for stable transformant eucaryotic host cells can be applied.

Examples of plasmids suitable for use in the practice of the invention include pCDM8, pCDM8neo, pcDNA1, pcDNA1neo, pMAMneo and Rc/CMV. Preferred plasmids include pCDM8neo, pcDNA1neo, pMAMneo and Rc/CMV.

Examples of viral expression vector systems suitable for the practice of the invention include those based upon retroviruses and those based upon baculovirus Autographa californica nuclear polyhedrosis virus.

Representative host cells comprising permanent cell lines suitable for use in the practice of the invention include CHO-K1 Chinese hamster ovary cells, ATCC-CCL-61; COS-1 cells, SV-40 transformed African Green monkey kidney, ATCC-CRL-1650; ATCC 20 murine pituitary cells; RIN-5F rat pancreatic β cells;
cultured insect cells, *Spodoptera frugiperda*; or yeast (*Saccharomyces*).

Example 7 contains a detailed explanation of preferred procedures used to express and secrete the 441-730 sequence. In that Example, the fragment is secreted as a homodimer held together by one or more disulfide bonds involving cysteine residues 459, 462 and 464. Expression of monomeric fragments useful as antithrombotics necessitates control be made of the disulfide bonding abilities of the monomers which is achieved most preferably by mutagenesis procedures as described in the aforementioned First Embodiment of the Invention.

The specific protocol used to generate the mutant vWF residue 441-730 fragment containing cysteine to glycine substitutions at each of residue positions 459, 462 and 464 is described in Example 9. The expression plasmid used therein was designated pAD4/Δ3C.

The specific protocol, adapted from that of Example 9, and which was used to generate the three mutant residue 441-730 fragments, each of which contains a different single Cys→Gly mutation (at positions 459, 462 or 464) is described in Example 11. The respective expression plasmids used therein were designated pAD4/G459, pAD4/G462 and pAD4/G464 (collectively "the pAD4/Δ1C plasmids"). Similar procedures may be used to produce mutant residue 441-730 fragments with Cys→Gly mutations at two of the three aforementioned positions.
Properties of the Polypeptides of the Second Embodiment

Homodimeric 116 kDa vWF Fragments

Example 7 below discloses the use of stably transformed CHO-K1 cells to express the unmutagenized residue 441-730 vWF subunit fragment. As set forth in Example 10 below, the unmutagenized fragment was also expressed in unstable COS-1 transformants.

SDS-polyacrylamide gel electrophoresis of secreted and immunoprecipitated proteins derived from CHO-K1 cells demonstrates that, under nonreducing conditions, the dominant vWF-derived polypeptide, detected by staining with Coomassie blue, has an apparent molecular weight of about 116,000 (Example 7). This result was confirmed by characterizing the polypeptides secreted by pAD4/WT transformed COS-1 cells (Example 12) using autoradiographs of $^{35}$S-labelled proteins. Under disulfide-reducing conditions (such as in the presence of 100 mM dithiothreitol) the 116 kDa fragment was no longer detected and the vWF-derived material appears as the expected 52/48 kDa monomer.

The apparent molecular weight of the recombinant 116 kDa polypeptide was consistent with the presence of said polypeptide as a homodimer of the 441-730 fragment. This homodimer carries also an amount of glycosylation equivalent to that observed in the 116 kDa polypeptide isolated by tryptic digestion of mature plasma (circulating) vWF. It is thus demonstrated that expression of the 441-730 fragment in the mammalian cell cultures of this invention favors the formation of the disulfide-dependent 116 kDa dimer thereof,
mimicking the structure seen in plasma. That the 116 kDa fragment so formed represents a correctly folded polypeptide was evidenced by its reaction (under nonreducing conditions) with conformation-dependent NMC-4 antibody. This antibody recognizes a properly assembled GPIb(α) binding site (Example 7). Reactivity with NMC-4 disappears under reducing conditions.

The dimeric 116 kDa fragment which is within the scope of the present embodiment and which contains two GPIb(α) binding sites supports ristocetin-induced platelet aggregation by virtue of its bivalent character. This was evidenced in Example 8 below.

Since it was demonstrated in the first embodiment (using bacterially-expressed vWF fragments) that cysteine residues 471 and 474 and also residues 509 and 695 are involved in intrachain bonds, the interchain bonds which stabilize the 116 kDa homodimer must be formed from one or more of residues 459, 462 and 464. It is further noted that since residues 459, 462 and 464 are in such close proximity in any monomer, there may be variation as to which particular residue or residues contribute the interchain disulfide bond or bonds which form the interpolypeptide contact in any particular mature vWF dimer or multimer, or recombinant 116 kDa fragment. Therapeutically-active populations of dimeric molecules can be generated according to the practice of the invention utilizing any of the possible combinations of interchain disulfide bonds.

It is noted that it is also possible that some structural folding or disulfide bond formation associated with the generation of therapeutically
active conformations of the recombinant 116 kDa dimers of the invention, or disulfide exchange therein, occurs after the polypeptides are secreted from a host cell.

Since there are also contained within the 441-730 vWF fragment potential binding sites for collagens, proteoglycans and glycosaminoglycans, the 116 kDa polypeptide is capable of performing a bridging function between a platelet and the subendothelium. This enables it to be used in a method for inducing platelet adhesion to surfaces such as, for example, vascular subendothelium. There is also provided a method of inducing platelet activation and/or aggregation which comprises contacting platelets with an effective amount of the recombinant 116 kDa polypeptide. Such a method is useful in the treatment of von Willebrand disease.

It is noted that as long as at least one of the one or more potential interchain disulfide bonds stabilizing the homodimer is left intact, and the amino acid sequences comprising the two GPIb(α) binding sites are preserved, that other regions of one or more of the two monomeric fragments thereof could be deleted, if necessary, to modify the therapeutic properties of the dimer.

52/48 kDa monomeric vWF fragments

An important aspect of the second embodiment of the invention is the provision of glycosylated 52/48 kDa monomeric fragments of the vWF subunit having substantial elements of normal tertiary structure.

Such fragments have a reduced tendency to form dimers
which tend to be unsuitable for use as antithrombotic therapeutics.

Following the above described procedures for site directed mutagenesis, residue 441-730 vWF fragments were produced in which one or more of cysteine residues 459, 462 and 464 were replaced with glycine residues. Examples 9, 10 and 11 below explain the mutagenesis and cell culture conditions necessary to create COS-1 cell transformants expressing these mutant vWF polypeptides. Examples 12 to 14 of the invention describe the properties of the molecules so derived in comparison with the recombinant 116 kDa polypeptide produced from pAD4/WT transformed COS-1 cells.

The vWF-derived polypeptides expressed by pAD4/Δ3C transformed COS-1 cells (containing the vWF 441-730 DNA sequence, but with each of cysteine codons 459, 462 and 464 thereof replaced by single glycine codons) were compared with the polypeptides secreted by pAD4/WT transformed COS-1 cells. To perform the comparisons, 35S-methionine-supplemented culture medium from each culture was subjected to immunoprecipitation using equal amounts of NMC-4 and RG-46 anti-vWF antibodies (Example 12) to collect the vWF-derived secreted proteins. The immunoprecipitated vWF polypeptides were then resolved by autoradiography of 35S-label on SDS polyacrylamide gels. No 116 kDa polypeptide could be detected in culture extracts of pAD4/Δ3C transformed cells under nonreducing conditions. Instead, under either reducing or nonreducing conditions, a band having an apparent molecular weight of 52 kDa was seen. In contrast, the pAD4/WT transformed COS-1 cells
produce under nonreducing conditions, as expected, a polypeptide of apparent molecular weight of 116 kDa.

The immunoprecipitation procedure was also repeated using only conformation-dependent NMC-4 antibody (Example 13). The major vWF-derived component isolated from the culture medium of pAD4/WT transformed cells again had an apparent molecular weight of 116 kDa under nonreducing conditions and 52 kDa under reducing conditions. A band of apparent 52 kDa molecular weight was detected under nonreducing conditions on gels of pAD4/Δ3C derived polypeptide material. As described in Example 13, reactivity with NMC-4 antibody is important evidence that the 52 kDa fragment detected in pAD4/Δ3C transformed cells possesses the tertiary structure of the natural residue 441-730 domain.

The immunoprecipitation procedure was also used to detect NMC-4 reactive vWF polypeptide produced by pAD4/Δ1C transformed COS-1 cells cultured under conditions similar to those for pAD4/WT and Δ3C transformants in the presence of 35S methionine. Immunoprecipitated proteins were run under reducing and nonreducing conditions in SDS-polyacrylamide gels and compared with vWF polypeptides produced by pAD4/WT and pAD4/Δ3C transformants (Example 14).

It was revealed that substitution of any one of cysteine residues 459, 462 or 464 by glycine results predominantly in a polypeptide having an apparent molecular weight of 52 kDa under nonreducing or reducing conditions, the formation of the 116 kDa species having been prevented.
The apparent molecular weight of 52 kDa for recombinant polypeptides derived from COS-1 cells transformed with either pAD4/Δ3C or pAD4/Δ1C plasmids is consistent with said polypeptides being monomers of the 441-730 fragment, while carrying also an amount of glycosylation equivalent to that seen in the 52 kDa polypeptide as isolated from tryptic digestion and reduction of mature plasma (circulating) vWF.

Unlike the dimeric polypeptides of apparent 116 kDa molecular weight, the monomeric 52 kDa polypeptides produced by pAD4/Δ1C and pAD4/Δ3C plasmids are unlikely to be capable of the bridging function associated with the dimer. Accordingly, there is provided a method of preventing platelet activation and/or aggregation which comprises contacting platelets with an effective amount of a mutant recombinant 52/48 kDa polypeptide which polypeptide shows at least a substantially reduced tendency to dimerize when compared with nonmutant (wild type) recombinant 52/48 kDa polypeptides.

There is further provided a method of preventing the adhesion of platelets to surfaces which comprises contacting platelets with an effective amount of a mutant recombinant 52/48 kDa polypeptide which shows at least a substantially reduced tendency to dimerize when compared with nonmutant recombinant 52/48 kDa polypeptides.

Contained within the 441-730 vWF fragment are potential binding sites for collagen (approximately residues 542-622) and glycosaminoglycans and proteoglycans (also within the residue 509-695 disulfide loop), in addition to the GPIbα binding
sites. It is probable because of steric considerations that a single fragment comprising residues 441-730 could not perform effectively as a bridging, potentially thrombotic, molecule. It is noted, however, that as long as the GPIb(α) binding domain of the 52/48 kDa monomer (consisting of approximately the primary sequence regions 474-488 and 694-708, and a tertiary domain thereof contributed in part by the 509-695 disulfide bond) is preserved, other regions (such as part of the heparin and collagen binding loop) of the said 52/48 kDa monomeric fragment could be deleted or altered, such as by proteolysis or by mutagenesis, if necessary, to modify or preserve the antithrombotic therapeutic properties thereof.

It is also possible that some structural folding or disulfide bond formation associated with the generation of therapeutically active conformations of the recombinant 52/48 kDa monomers of the invention, or disulfide exchange therein, occurs after the polypeptides are secreted from a host cell.

Limitation of the Glycosylation of vWF-Derived Polypeptides to Enhance Therapeutic Activity

von Willebrand factor and platelet glycoprotein Ib(α) are glycoproteins, that is, proteins to which carbohydrate molecules (such as sugars) are attached. In the case of von Willebrand factor, this natural process of adding carbohydrate (referred to as glycosylation) substantially increases the molecular weight of the protein. For example, with respect to the tryptic fragment of the mature vWF subunit which consists of residues 449-728, the apparent molecular
weight rises from about 38 kDa to 52 kDa in humans as a result of said glycosylation.

Glycosylation of newly synthesized polypeptides is much more complex in eucaryotic cells (such as mammalian cells) than in bacterial cells. Glycosylation has been found to be particularly common in protein species which serve as membrane receptors, such as GPIb(α), and in proteins which interact therewith (such as vWF).

By way of background, glycosylation is typically accomplished in mammalian cells in several stages beginning soon after the nascent polypeptide appears on the ribosome and continuing as the protein is further processed for ultimate insertion into the cell membrane, or for secretion from the cell. Since glycosylation is so important to the function of many glycoproteins (see Wagner, D.D. et al., J. Cell Biol., 102, 1320-1324 (1986) concerning certain possible functions with respect to vWF), the role of glycosylation in the GPIb(α)-binding activity of the residue 449-728 region of the mature von Willebrand factor subunit was investigated. As demonstrated herein the therapeutic activity of the vWF 116 kDa dimer can be enhanced by restricting the glycosylation thereof. This indicates that the activity of 52/48 kDa monomers should also be similarly enhanced.

Any suitable means can be used to restrict the glycosylation of the vWF 116 kDa dimers or of the 52/48 kDa monomers.
By way of background, it is noted that glycosylation of the 52/48 tryptic fragment of circulating mature von Willebrand factor subunit has been determined to occur predominantly at residue positions 468 (asparagine); 500 and 723 (serine); and 485, 492, 493, 705, 714 and 724 (threonine). Titani, K. et al., *Biochemistry*, 25, 3171-3184 (1986). Glycosylation of asparagine is N-linked (from the side chain amide group). Serine and threonine hydroxyl groups present O-linked glycosylation sites. The present invention encompasses modification of glycosylation at both N- and O-linked sites.

Tunicamycin, an antibiotic which may be isolated from cultures of *Streptomyces* has been demonstrated to inhibit the glycosylation of proteins in eucaryotic cells. Duskin, D. et al., *J. Biol. Chem.*, 257(6), 3105-3109 (1982). Specifically, tunicamycin inhibits the synthesis of N-type glycosidic linkages (at asparagine N-linked sites). See Mahoney, W.C. et al., *J. Chromatog.*, 198, 506-510 (1980). Accordingly, the treatment of eucaryotic cells with tunicamycin provides for an effective system in which to modify glycoproteins that are produced therein.

Following the procedure of Example 15, stable CHO-K1 transformants containing pAD5/WT plasmids and capable of secreting the recombinant 116 kDa vWF fragment (see Example 1) were cultured in the presence of tunicamycin. After about 36 hours, the culture medium was harvested and concentrated. The concentrated culture medium, in which the dominant vWF-derived polypeptide species has an apparent molecular weight of 116 kDa, was tested in ristocetin-induced
platelet aggregation assays (see Example 9) and compared with culture medium from untreated cell cultures (generating polypeptides with normal N-linked glycosylation). It was demonstrated that the tunicamycin induced limitation on N-linked glycosylation of the secreted 116 kDa vWF fragment substantially increased its ability to support ristocetin-induced platelet aggregation.

For the purpose of inhibiting N-linked glycosylation, it is preferred in the practice of this invention to add to the culture medium of cells expressing vWF-derived polypeptide a concentration of tunicamycin between about 0.3 and about 1.5 μg/ml. Below about 0.3 μg/ml, the action of the antibiotic tends to result in a heterogeneous population of differentially cleaved polypeptides. This effect is not expected to be significantly lessened by longer exposure of the host cells to antibiotic-containing medium since the nascent vWF polypeptides are likely only to be processed by glycosylating enzymes for a limited period of time after translation. Above approximately 1.5 μg/ml, there were signs of toxicity with respect to CHO-K1 cells. It is believed that this was caused by inhibition of glycosylation of CHO-K1 proteins, such glycosylation being necessary for cell function and growth. Although the range of suitable tunicamycin concentrations may be different under different cell culture conditions, or with different host cell lines, the above guidelines can be readily used to ascertain with respect to other cell lines appropriate tunicamycin incubation conditions.
The Role of Sialic Acid-containing Carbohydrate Side Chains in the Binding of the 52/48 vWF Fragment to Platelets

One of the most important types of carbohydrate which is found on both N- and O-linked carbohydrate side chains of the 52/48 tryptic fragment is sialic acid. Sialic acid is negatively charged and contributes to regions of net negative change on the surface of vWF multimers and also of platelet GPIb(α) receptors. Sialic acid facilitates mutual repulsion of vWF and GPIb(α) under non-injury conditions. In fact, platelets and circulating vWF normally coexist in the blood without any interaction occuring, although vWF bound to the subendothelium, presumably as a result of chemical or physical changes induced by injury, binds to platelets.

The vWF-platelet GPIb(α) interaction can be demonstrated in vitro in the presence of certain mediators such as the positively charged glycopeptide ristocetin, or following chemical manipulation of the vWF molecule itself, as by removal of terminal negatively charged sialic acid residues from carbohydrate side chains. DeMarco, L. et al., J. Clin. Invest., 68, 321-328 (1981). Sialic acid residues are found in carbohydrate side chains which are attached to serine and threonine sites (O-linked) and also asparaginase (N-linked) sites in the residue 449-728 vWF fragment.

The effect of tunicamycin in enhancing the therapeutic capability of the 116 kDa fragment results in part from limiting the sialic acid content of the 116 kDa dimer. This effect should be equally applicable to 52/48 kDa monomers. Accordingly, the
treatment with tunicamycin of host cells containing expression plasmids which produce monomeric 52/48 kDa vWF fragments (such as pAD4/Δ3C or pAD4/Δ1C, Examples 9 and 11) will cause to be expressed therefrom antithrombotic therapeutics with increased GPIb(α) binding activity.

Accordingly this invention encompasses the process of treating a eucaryotic host cell which contains a DNA sequence encoding the 449-728 tryptic vWF fragment with tunicamycin for the purpose of limiting the glycosylation of said fragment, or of dimers thereof.

This invention also encompasses additional ways to restrict the glycosylation of vWF-derived polypeptides for the purpose of improving the therapeutic utility thereof.

(A) It is noted that there are numerous enzymes which can be used to cleave carbohydrate side chains (including N- or O-linked) from glycoproteins. Representative examples include (1) O-glycanase®, an endo-α-N-acetyl-galactosaminidase, which cleaves O-linked sugars where there is a gal-β-(1,3)gal NAc core disaccharide linked to a serine or threonine residue; (2) N-glycanase®, an N-glycosidase F, peptide- N°[N-acetyl-β-glucosaminyl]asparagine amidase, which hydrolyzes asparagine-linked oligosaccharides; and (3) gal-β-1,4-GlcNAc-α-2,6-sialyl transferase, which can be used to modify sialic acid sites, all from Genzyme Co., Boston, MA,
or endo-H and endo-F from Sigma Chemical Co., St. Louis, MO.

(B) Example 16 describes the production by site directed mutagenesis of mutant polypeptides patterned upon a parent polypeptide which comprises the amino acid sequence of that fragment of mature von Willebrand factor which begins approximately at residue 449 (valine) and ends approximately at residue 728 (lysine). It is taught in Example 16 that particular codons encoding serine, threonine, and also asparagine residue which are or are potentially sites of O- or N-linked glycosylation respectively (for the parent polypeptide encoded by a vWF cDNA) can be deleted or replaced with codons for other amino acids thereby enabling the expression in host cells and secretion therefrom of a polypeptide having less glycosylation (including sialated carbohydrate) than the parent vWF polypeptide.

As taught in Examples 7, 9 and 11, control over whether the expressed polypeptide is dimeric or monomeric is effected by mutation of one or more of cysteine codons 459, 462 and 464. Monomeric polypeptides so derived are useful antithrombotics whereas the dimeric forms are useful in treating hemorrhage in patients with von Willebrand disease. It is noted that the same secondary and tertiary structural factors previously described for selecting suitable replacement amino acids for cysteine residues
may be applied to replace the serine, threonine and asparagine residues which are glycosylation sites. In addition, and for the purpose of designing mutant vWF-derived polypeptides, although serine, threonine, and asparagine are considered suitable replacements for most cysteine residues, the possibility must be considered that cys → thr, cys → ser or cys → asn substitutions will introduce into the vWF-derived polypeptide new glycosylation sites resulting in polypeptides with increased carbohydrate content.

It is also noted that mutant polypeptides derived from the 449-728 region of the mature vWF subunit can be designed to possess substantially increased carbohydrate content by using site directed mutagenesis procedures to introduce additional serine, threonine and asparagine codons into a DNA within a host cell capable of glycosylating the polypeptide and then secreting it.

**Use of the von Willebrand Factor Signal Peptide to Effect Secretion from Host Cells of Non-vWF Derived Polypeptides**

The present invention provides also a process for producing from an encoding DNA sequence biologically active monomers and dimers corresponding approximately to the residue 449-728 sequence of the mature von Willebrand factor subunit, which polypeptides are secreted from host cells. Of central importance to the success of this process is the assembly of a vWF DNA sequence to which is also attached a DNA sequence encoding the vWF signal peptide. Recognition of the signal sequence by cellular components enables the vWF polypeptide to be secreted from the cell instead of accumulating therein as a substantially insoluble
aggregation of polypeptides. Proteins trapped in inclusion bodies are generally believed to demonstrate improper folding and disulfide bonding. See Williams, D.C. et al., *Science*, 215, 687 (1982).

In order for the recombinant polypeptide representing vWF subunit residues 441 to 730 to be secreted from a host cell, it is necessary that the nascent polypeptide which combines the signal peptide and mature vWF subunit sequence be recognised by the endoplasmic reticulum and cellular components such as translocation receptors and signal peptidase which are necessary to the process of secretion. Proper recognition of the carboxy terminal end of the signal peptide by signal peptidase is generally required. For the purpose of enhancing the secretion from host cells of the recombinant 441-730 vWF fragment or other unrelated therapeutic polypeptides, there may also be inserted between the DNA encoding the vWF signal peptide and the DNA encoding the structural sequence of the therapeutic polypeptide a small spacer DNA sequence.

Preferred examples of spacer DNA include sequences encoding from about one to about ten of the amino acid residues which comprise the amino terminal sequence region of the vWF propeptide. Particularly preferred as spacer cDNA sequences are those which encode

\[ \text{NH}_2\text{-Ala-Glu-Gly-CO}_2\text{H}, \]
\[ \text{NH}_2\text{-Ala-Glu-Gly-Thr-CO}_2\text{H}, \text{ or} \]
\[ \text{NH}_2\text{-Ala-Glu-Gly-Thr-Arg-CO}_2\text{H}, \]

which represent the first 3, 4 and 5 amino acid residues of the amino terminal region of the vWF propeptide.
Example 17 teaches conditions under which such combined constructs may be expressed in host cells. As depicted in Example 17 of the invention, the vWF signal peptide contains a substantially hydrophobic region (as is true of most signal peptides) whereas the amino terminal region of the vWF propeptide is substantially hydrophilic.

Proper recognition by signal peptidases of target cleavage sites generally requires semipolar or polar regions adjacent to or in conjunction with the carboxy terminal region of the signal peptide. von Heijne, G., J. Mol. Biol., 184, 99-105 (1985).

The DNA sequence used in the practice of this invention to cause secretion of the 52/48 kDa domain of the vWF subunit provides such a polar domain by connecting to the signal peptide a spacer derived from the vWF propeptide (Ala-Glu-Gly) followed by the highly polar Arg_{41} Arg_{42} residues of the mature subunit sequence.

This aspect of the invention is particularly important with respect to the expression and secretion of therapeutic fragments of polypeptides. Such fragments do not include the amino terminal region of the entire polypeptide. The amino terminus would normally present a hydrophilic domain which is positioned directly adjacent to the carboxy terminus of the signal peptide. In such cases, a semipolar or polar spacer (such as ala-glu-gly of vWF) may be caused to be inserted between the signal sequence and the sequence for the therapeutic polypeptide fragment to facilitate recognition as a proper signal peptidase.
cleavage site. Alternatively, and if the polypeptide fragment's activity is unaffected, the exact residue position which comprises the amino terminus of said cloned therapeutic polypeptide fragment may be selected so as to commence a region of hydrophilic residues which will form a recognition sequence. The substantially hydrophilic character of residue 441-450 region of the 52/48 kDa fragment indicates that the fragment may be successfully expressed within and secreted from eucaryotic cells without use of a spacer between the signal peptide and the mature subunit sequence.

The use of preferred species of spacer polypeptides (such as the first 3, 4 or 5 residues of the vWF propeptide) is advantageous in that it causes to remain attached to the therapeutic polypeptide upon secretion from the cell only a biologically insignificant sequence of foreign amino acids unlikely to affect the function of the therapeutic polypeptide.

The following information is provided to facilitate selection of semipolar and polar spacer sequences useful in the practice of the invention.

It is possible to predict the extent of relative hydrophobic or hydrophilic character which a particular peptide sequence will exhibit when present within larger polypeptides. One such model is the relative hydrophobicity/hydrophilicity index as described by Kyte, J. et al., J. Mol. Biol., 156, 105-132 (1982). An overall index value is assigned based on individual residue contributions and the position of the particular amino acid residues within the peptide. The
maximum value of hydrophobicity described therein is +4.5 (equivalent to isoleucine). The maximum value of hydrophilicity described therein is -4.5 (equivalent to arginine). In the practice of the present invention, a spacer peptide is considered semipolar or polar if according to the method of Kyte, J. et al., supra, it possesses an overall index value of between approximately 0 and approximately -4.5. It is noted however that a very short, slightly hydrophobic spacer may nonetheless prove functional if the adjacent therapeutic polypeptide sequence is highly polar.

Representative index values for spacer peptides useful in the practice of the invention are as follows. Subscript numbers refer to residue positions within the mature vWF subunit sequence which can be seen to alter significantly the relative hydrophilicity of the combined sequence region.

(A) Ala-Glu-Gly
(B) Ala-Glu-Gly-Thr
(C) Ala-Glu-Gly-Thr-Arg
(D) Ala-Glu-Gly-Arg<sub>441</sub>-Arg<sub>442</sub>
(E) Ala-Glu-Gly-Arg<sub>441</sub> to Lys<sub>447</sub>
(F) the first ten (amino terminal) residues -1.45

With respect to the expression of therapeutic polypeptides derived from von Willebrand factor or other proteins in which recombinant DNA-directed methods are used to create a host cell transformed with an expression plasmid or viral expression vector containing an appropriate DNA, it is generally accepted that a variety of eucaryotic signal peptides are suitable. In addition, amino acid sequence subsets of
signal peptides which supply the necessary hydrophobic domain thereof are useful in the practice of the invention.

Preferred as additional polypeptides which may be successfully secreted from host cells by constructing a DNA sequence encoding the target polypeptide and a vWF signal peptide sequence are polypeptides comprising "A" type sequence domains. Preferred as additional polypeptides which may be successfully secreted from host cells by constructing a DNA sequence encoding the target polypeptide and a vWF signal peptide/propeptide sequence are also polypeptides comprising "A" type sequence domains.

"A" type domains have originated from gene duplication of a common structural genetic element with the result that they share substantial amino acid sequence homology (greater than approximately 15 to 20%) with the region of the mature vWF subunit between approximately residues 500 and 700. Mancuso, D.J. et al., J. Biol. Chem., 264(33) 19514-19527 (1989).

Representative of such proteins are complement factor B, complement component C2, cartilage matrix protein, \( \alpha_1 \)-collagen type VI, \( \alpha \) subunits of leucocyte adhesion receptors Mac-1, and LFA-1, VLA-1 and VLA-2. The 2,050 residue mature von Willebrand subunit itself contains two other "A" domains, \( A_2 \) (approximately residues 710-910) and \( A_3 \) (approximately residues 910-1110).

**Antibodies with Therapeutic Activity**

Antibodies, and particularly conformation dependent antibodies, are powerful tools for analyzing
the structure and function of macromolecules. By blocking macromolecular interactions, antibodies can also have important therapeutic utility.

Accordingly, this invention includes within its scope an antibody which is specific for the vWF subunit, or any polypeptide containing a subset thereof which antibody is made by a process which involves immunizing animals with a polypeptide patterned upon the mature vWF subunit sequence between approximately residue 441 and residue 730 thereof, and having less tendency than the polypeptide upon which it is patterned to form interchain disulfide bonds owing to deletion or replacement, of one or more of cysteine residues 459, 462 or 464 of the pattern sequences. Further diagnostic or therapeutically useful antibodies can be generated against polypeptides so patterned upon the above stated sequence region and in which cysteine residues 509 and 695 form a disulfide bond, thereby recreating important domains of tertiary structure.

**Therapeutic compositions**

One or more of the polypeptides of the present invention can be formulated into pharmaceutical preparations for therapeutic, diagnostic, or other uses. To prepare them for intravenous administration, the compositions are dissolved in water containing physiologically compatible substances such as sodium chloride (e.g. at 0.35-2.0 M), glycine, and the like and having a buffered pH compatible with physiological conditions, which water and physiologically compatible substances comprise a pharmaceutically acceptable carrier.
With respect to the monomeric 52 kDa polypeptides of the invention having at least a substantially reduced tendency to dimerize, the amount to administer for the prevention or inhibition of thrombosis will depend on the severity with which the patient is subject to thrombosis, but can be determined readily for any particular patient.

With respect to the recombinant 116 kDa polypeptide of the invention, or other dimeric polypeptide subfragments thereof, the amount to administer for the treatment of von Willebrand disease will depend on the severity with which the patient is subject to hemorrhage, but can be determined readily for any particular patient.

Examples

The following Examples are representative of the practice of the invention.

I. Construction of vWF Polypeptides Suitable to Carry IIb-Type Mutations

Example 1 - Expression of a mutant cysteine-free mature von Willebrand factor subunit fragment having an amino terminus at residue 441 (arginine) and a carboxy terminus at residue 733 (valine)

Preparation of a cDNA Clone from pre-pro-von Willebrand Factor mRNA

A cDNA clone encoding the entire von Willebrand factor gene (for the pre-propeptide) was provided by Dr. Dennis Lynch, Dana-Farber Cancer Institute, Boston,
MA and was prepared as described in Lynch, D.C. et al., *Cell*, 41, 49-56 (1985). It had been deemed probable that the size of vWF mRNA would likely exceed that of human 28S type rRNA. Accordingly, total RNA from endothelial cells (the major source of plasma vWF) was sedimented in sucrose gradients, with RNA larger than 28S being selected for construction of a cDNA library.

This enriched fraction was further purified using two separate cycles of poly(u)-Sephadex® chromatography to select for RNA species (mRNA) having 3' polyadenylated ends. Lynch et al., supra, estimated the prevalence of vWF mRNA in this fraction at about 1 in 500, which fraction was used to generate a cDNA library of approximately 60,000 independent recombinants.

To generate the cDNA library, standard techniques were used. The mRNA population was primed using an oligo (dT) primer, and then transcribed with a reverse transcriptase. The RNA strands were then removed by alkaline hydrolysis, leaving cDNA anticoding strands (equivalent to transcribed strands) which were primed by hairpin looping for second strand synthesis using DNA polymerase I. The hairpin loop was removed with S1 nuclease and rough ends were repaired with DNA polymerase I.

GC tailing, Maniatis, T. et al., *Molecular Cloning*, 2nd ed., v.1, p.5.56 (1987), was then used to anneal the cDNA into plasmid vector pBR322. Oligo(dC) tails were added to the cDNA fragments with terminal transferase and were annealed to oligo(dG) tailed
pBR322. The plasmids were transformed into ampicillin sensitive *E. coli*, strain HB101 for propagation. Suitable clones were identified after screening with $^{32}$P-labelled cDNA prepared as reverse transcriptase product of immunopurified vWF polysomes. Positive clones were subcloned into pSP64 (Promega Co., Madison, WI).

**Primer Directed Amplification of cDNA**

cDNA representing the full length pre-pro-vWF gene from pSP64 was subjected to enzymatic amplification in a polymerase chain reaction. Based upon the established nucleotide sequence of the pre pro-vWF gene, Bonthron, D. et al. *Nucl. Acids Res.*, 14(17), 7125-7127 (1986); Mancuso, D. et al., *J. of Biological Chemistry*, v.264(33), 19514-19527 (1989) oligonucleotides flanking the region of interest (designated (1), SEQ ID NO: 2, and (2), SEQ ID NO: 3) were prepared. All oligonucleotides used herein were synthesized by the phosphoramidite method, Sinha, et al., *Tetrahedron Letters*, 24, 5843 (1983), using a model 380B automated system, Applied Biosystems, Foster City, CA.

Oligonucleotide (1) (SEQ ID NO: 2)

5'-ACGAATTC CGG CGT TTT GCC TCA GGA3'
EcoRI Arg441 Gly446

Oligonucleotide (2) (SEQ ID NO: 3)

3'-GG GAC CCC GGG TTC TCC TTG AGG TAC CAT TCGAAGG5'
5'-ctg ggg ccc aag agg aac tcc atg gta agcttc3'
Leu725 Met732 Val733 HindIII
The oligonucleotides overlap the ends of the coding region for that fragment of the mature vWF subunit which can be produced by digestion with trypsin and which begins with residue 449 (valine) and ends with residue 728 (lysine). Oligonucleotide (1) corresponds to coding strand DNA (analogous with mRNA) for amino acid positions 441 to 446 and adds an EcoRI restriction site 5' to the codon for amino acid 441. Oligonucleotide (2) corresponds to the non-coding strand (transcribed strand) of mature vWF DNA for amino acids positions 725-733 and adds a HindIII restriction site 3' to the codon for amino acid 733. The coding strand complementary to (2) is shown in lower case letters.

Using the above oligonucleotides with the full length cDNA as template, a cDNA fragment corresponding to mature vWF residues Nos. 441-733, and containing EcoRI and Hind III linkers, was then synthesized in a polymerase chain reaction following the method of Saiki, R.K. et al. Science, 239, 487-491 (1988).

The procedure utilizes a segment of double-stranded vWF cDNA, a subsegment of which is to be amplified, and two single-stranded oligonucleotide primers (in this case oligonucleotides (1), (2)) which flank the ends of the subsegment. The primer oligonucleotides (in the presence of a DNA polymerase and deoxyribonucleotide triphosphates) were added in much higher concentrations than the DNA to be amplified.

Specifically, PCR reactions were performed with a DNA thermal cycler (Perkin Elmer Co., Norwalk, CT/Cetus
Corporation, Berkeley, CA) using Taq polymerase (Thermus aquaticus). The reactions were run in 100 μl volumes containing 1.0 μg of pre-pro-vWF cDNA, 1.0 μg of each synthetic oligonucleotide primer, and buffer consisting of 50 mM KCl, 10 mM Tris·HCl (pH 8.3), 1.5 mM MgCl₂, 0.1% gelatin (BioRad Co., Richmond, CA) and 200 mM of each dNTP. PCR conditions were 35 cycles of 30 seconds at 94°C, 30 seconds at 52°C and 1 minute at 72°C. Amplified fragments were then purified and isolated by electrophoresis through a 2% agarose gel, Maniatis et al., Molecular Cloning, A Laboratory Manual, 164-170, Cold Spring Harbor Lab., Cold Spring Harbor, NY (1982).

The vast majority of polynucleotides which accumulate after numerous rounds of denaturation, oligonucleotide annealing, and synthesis, represent the desired double-stranded cDNA subsegment suitable for further amplification by cloning.

For some experiments, cDNA corresponding to the mature vWF fragment beginning at amino acid sequence position 441 and ending at position 733 was prepared and amplified directly from platelet mRNA following the procedure of Newman, P.J. et al. J. Clin. Invest., 82, 739-743 (1988). Primer nucleotides No. 440 and 733 were utilized as before with the resulting cDNA containing EcoRI and HindIII linkers.

**Insertion of cDNA into M13mp18 Cloning Vehicle**

The resultant double stranded von Willebrand factor cDNA corresponding to the amino acid sequence from residue 441 to 733 was then inserted, using EcoRI
and HindIII restriction enzymes, into the double stranded replicative form of bacteriophage M13mp18 which contains a multiple cloning site having compatible EcoRI and HindIII sequences.

M13 series filamentous phages infect male (F factor containing) E.coli strains. The infecting form of the virus is represented by single stranded DNA, the (+) strand, which is converted by host enzymes into a double stranded circular form, containing also the minus (−) strand, which double stranded structure is referred to as the replicative form (RF). The ability to isolate a stable single stranded (+) form of the virus is particularly useful to verify the integrity of any cloned sequences therein. See Messing, J., Meth. Enzymology, 101, 20-78 (1983); Yanish-Perron, C. et al., Gene, 33, 103-109 (1985).

Accordingly, the vWF cDNA insert was completely sequenced using single-stranded dideoxy methodology (Sanger, F. et al. Proc. Natl. Acad. Sci USA, 74, 5463-5467 (1977)), utilizing the single-stranded (+) form of M13mp18, to confirm that the vWF cDNA fragment contained the correct coding sequence for mature vWF subunit residues 441-733.

Site-Directed Mutagenesis to Replace Cysteine Residues

Cysteine residues 459, 462, 464, 471, 474, 509, and 695, within the mature vWF fragment corresponding to amino acids 441 to 733, were replaced with glycine residues by substitution of glycine codons for cysteine codons in the corresponding cDNA. In order to accomplish this, oligonucleotides (see Sequence Listing
ID NOS: 5-8) encompassing the region of each cysteine codon of the vWF cDNA were prepared as non-coding strand (transcribed strand) with the corresponding base substitutions needed to substitute glycine for cysteine. The oligonucleotides used were as follows:

Oligonucleotide (3) (SEQ ID NO: 4)

3′GGA CTC GTG CCG GTC TAA CCG GTG CAA CTA CAA CAG5′
5′cct gag gac ggc cag att ggc cac gat gat gtt gtc3′
Pro Glu His Gly Gln Ile Gly His Gly Asp Val Val
459 462 464
(simultaneously replacing cysteines 459, 462, 464).

Oligonucleotide (4) (SEQ ID NO: 5)

3′TTG GAG TGG CCA CTT CCG CCG GTC CTC GGC5′
5′aac ctc acc ggt gaa gcc ggc cag gag ccg3′
Asn Leu Thr Gly Glu Ala Gly Gln Glu Pro
471 474
(simultaneously replacing cysteines 471, 474)

Oligonucleotide (5) (SEQ ID NO: 6)

3′CTA AAG ATG CCG TCG TCC G5′
5′gat ttc tac ggc agc agg c3′
Asp Phe Tyr Gly Ser Arg
509
(replacing cysteine 509)

Oligonucleotide (6) (SEQ ID NO: 7)

3′TCG ATG GAG CCA CTG GAA CGG5′
5′agc tac ctc ggt gac ctt gcc3′
Ser Tyr Leu Gly Asp Leu Ala
695
(replacing cysteine 695)

Hybridizing oligonucleotides are shown in capital letters and are equivalent to the transcribed strand (non-coding DNA). The equivalent coding strand is
shown in lower case letters with the corresponding amino acids shown by standard three letter designation. (for designations see Table 1)

As elaborated below, cysteines 459, 462 and 464 were replaced simultaneously using oligonucleotide (3). Cysteine residues 471 and 474 were then replaced simultaneously using oligonucleotide (4). Cysteine residues 509 and 695 were then replaced individually using oligonucleotides (5) and (6) respectively.

The cysteine to glycine cDNA substitutions were accomplished following the procedure of Kunkel, T.A., Proc. Natl. Acad. Sci. USA, 82, 488-492 (1985) which procedure repeats a series of steps for each oligonucleotide and takes advantage of conditions which select against a uracil containing DNA template:

(A) M13mp18 phage, containing wild type vWF cDNA corresponding to amino acid positions 441 to 733, is grown in an E.coli CJ236 mutant dut−ung− strain in a uracil rich medium. Since this E.coli strain is deficient in deoxyuridine triphosphatase (dut−), an intracellular pool of dUTP accumulates which competes with dTTP for incorporation into DNA. (see Shlomai, J. et al. J. Biol. Chem., 253(9), 3305-3312 (1978). Viral DNA synthesized under these conditions includes several uracil insertions per viral genome and is stable only in an E.coli strain
which is incapable of removing uracil, such as (ung⁻) strains which lack uracil glycosylase. Uracil-containing nucleotides are lethal in single stranded (⁺) M13mp18 DNA in ung⁺ strains due to the creation of abasic sites by uracil glycosylase.

(B) Single-stranded (⁺) viral DNA is isolated from culture media in which phage were grown in E.coli strain CJ236 dut⁻ung⁻. The single stranded (⁺) form of the virus contains the specified vWF cDNA at its multiple cloning site which cDNA is equivalent to the nontranscribed vWF DNA strand.

(C) Oligonucleotide (3), which contains codon alterations necessary to substitute glycines for cysteines at positions 459, 462 and 464, is then annealed in vitro to single stranded (⁺) phage DNA. Generally, a wide range of oligonucleotide concentrations is suitable in this procedure. Typically 40 ng of oligonucleotide was annealed to 0.5-1.0 µg M13mp18 phage (⁺) DNA.

(D) All missing sequence of the M13mp18(⁻) strand is then completed in vitro using T₇ DNA polymerase and T₄ DNA ligase in a dTTP rich environment thereby generating a transcribable vWF cDNA sequence
corresponding to amino acid positions 441 to 733 of the mature vWF subunit.

(E) The double stranded M13mp18 phage, now containing a thymine normal (\(\ast\)) strand and a (\(^+\)) strand with several uracil substitutions, is transformed into a wild type E.coli XL-1 Blue (Stratagene, La Jolla, CA) strain which contains normal levels of uracil glycosylase and deoxyuridine triphosphatase.

(F) Uracil glycosylase and other enzymes present in the new host initiate destruction of the uracil-containing (\(^+\)) strand of the double-strand phages, leading after replication in the host of remaining phage (\(\ast\)) strand DNA to the presence of stable thymine-normal double stranded (RF) DNA which reflects the glycine mutations induced by the oligonucleotide.

(G) Steps (A) to (F) of the above process are then repeated for each of oligonucleotides (4), (5) and (6) until each successive cysteine codon of the vWF sequence within the M13mp18 phage has been replaced by a glycine codon.

(H) Upon completion of mutagenesis procedures the sequence of the vWF cDNA insert was reconfirmed using
the single stranded DNA dideoxy method. (Sanger, F. et al., supra)

Construction of Expression Plasmids

The double stranded vWF cDNA fragment containing 7 site-specific cysteine to glycine mutations is then removed from M13mp18 phage by treatment with EcoRI and HindIII restriction endonucleases, after which the ends of the fragment are modified with BamHI linkers (Roberts, R.J. et al. Nature, 265, 82-84 (1977)) for cloning into a high efficiency E.coli expression vector. The particular expression vector chosen is plasmid pET-3A, developed by Rosenberg, A.H. et al. Gene, v.56, 125-135, (1987) and which is a pBR322 derivative containing a high efficiency (φ10) T7 transcription promoter directly adjacent to the BamHI linker site. When containing the above-specified fragment of mutant vWF cDNA, the pET-3A vehicle is referred to as "p7E" or p7E expression plasmid.

A second pET-3A-derived expression plasmid (designated p7D) was constructed containing the identical vWF coding sequence cloned into the plasmid in the opposite orientation. p7D should be unable to express the vWF polypeptide fragment.

A third expression plasmid (pJD18) contains wild type 52/48 tryptic vWF fragment cDNA encoding the vWF amino acid sequence between residues 441 and 733, (with 7 cysteines) in the same pET-3A vector.

The p7E (or p7D and pJD18) expression plasmids were then cloned into an ampicillin sensitive E.coli
strain, BL21(DE3), Novagen Co., Madison WI, according to a well established protocol Hanahan, D., *J. Mol. Biol.*, 166, 557-580 (1983). Strain BL21(DE3) is engineered to contain a gene for T7 RNA polymerase so that the vWF insert can be transcribed with high efficiency.

**Expression of Mutant vWF Polypeptides**

Three separate samples of *E. coli* strain BL21(DE3) containing respectively p7E, p7D or pJD18 expression plasmids were inoculated into 5-6 ml of 2X-YP growth medium containing 200 μg/ml of ampicillin, and grown overnight at 37°C to create fully grown cultures. 2X-YP growth medium contains, per liter of water, 10 gm Bacto-tryptone, 10 gm yeast extract and 5 gm NaCl.

Five ml of each overnight culture was then inoculated into 500 ml of 2X-YP medium, again containing 200 μg/ml of ampicillin and grown for 2 hours at 37°C with shaking.

After the 2 hour incubation period, the cultures were induced for protein expression by addition of isopropyl-beta-d-thiogalactopyranoside to a concentration of 5 mM. The incubation was then continued for 3 hours at 37°C.

A high level of expression of vWF polypeptide was obtained with p7E and pJD18 resulting in the generation of cytoplasmic granules or "inclusion bodies" which contain high concentrations of vWF polypeptide in essentially insoluble form. Solubilization of vWF polypeptide was accomplished according to the following procedure. As explained in Example 2, p7E and pJD18

The cells were harvested by centrifugation at 4000 g for 15 minutes in a JA-14 rotor at 4°C. The pelleted cells were washed in 50 ml of ice cold buffer (0.1 M NaCl, 10 mM Tris pH 9.0, 1 mM EDTA) and repelleted by centrifugation at 4000 g at 4°C.

The cell pellets from p7E, p7D and pJD18 cultures were each redissolved in 5 ml of lysing buffer and kept ice-cold for 30 minutes. The lysing buffer comprises a solution of sucrose 25%(w/v), 1 mM phenylmethylsulfonylfluoride (PMSF), 1 mM ethylene diaminetetraacetic acid (EDTA), 2 mg/ml lysozyme and 50 mM Tris hydrochloride, adjusted to pH 8.0.

After the 30 minute incubation, aliquots of 1.0 Molar MgCl₂ and MnCl₂ were added to make the lysing solution 10 mM in each cation. Sixty μg of DNAseI (Boehringer-Mannheim) was then added and the incubation was continued at room temperature for 30 minutes.

Twenty ml of buffer No. 1 (0.2 M NaCl, 2 mM EDTA, and 1% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 1% (w/v) Non-ident 40, and 20 mM Tris hydrochloride, pH 7.5) was then added to the incubation mixture. The insoluble material was
pelleted by centrifugation at 14,000 g (12,000 rpm in a
JA-20 rotor) for 30 minutes at 4°C.

The relatively insoluble pelleted material derived
from each culture (which contains the desired
polypeptides except in the case of p7D) was washed at
25°C in 10 ml of buffer No. 2 (0.5% (w/v) Triton X-100
surfactant, 2 mM EDTA, 0.02 M Tris hydrochloride, pH
7.5) and vortexed extensively. The suspension was
centrifuged at 14,000 g for 30 minutes at 4°C and the
supernatant was then discarded. The process of
resuspension of the pelleted material in buffer No. 2,
vortexing and centrifugation was repeated twice.

Each pellet was then washed in 5 ml of buffer No.
3 (0.02 M Tris hydrochloride, pH 7.5, and 2 mM EDTA) at
25°C and vortexed extensively. The suspension was then
centrifuged at 4°C for 30 minutes at 14,000 g after
which the supernatant was discarded leaving a pellet of
inclusion body derived material (the "wet pellet") with
a clay-like consistency (With respect to the following
final steps, and in replacement therefor, see also
Example 20 which presents an additional improved
procedure).

The insoluble pellet was slowly redissolved in an
8 Molar urea solution held at room temperature for 2
hours, after which solubilization was continued
overnight at 4°C. The urea-soluble material was
extensively dialyzed against a solution of 0.15 M NaCl
containing 20 mM Hepes (N-[2-hydroxyethyl]piperazine-N-
[2-ethanesulfonic acid]) (pH 7.4) ("Hepes-buffered
saline") at 4°C.
The solubilized peptide extracts were assayed for purity (Example 2), used in vWF binding inhibition assays (Example 3) or subject to further purification. Further purification steps should not be delayed and the samples should remain cold.

The cysteine-free vWF polypeptide (comprising subunit positions 441 to 733) constitutes more than 75% of the material solubilized from the inclusion bodies according to the above procedure. Further purification of the cysteine-free mutant vWF polypeptide was accomplished by redialyzing the partially purified peptide extract against 6 M guanidine·HCl, 50 mM Tris·HCl, pH 8.8 followed by dialysis against 6 M urea, 25 mM Tris·HCl, 20 mM KCl, 0.1 mM EDTA, pH 8.0. The extract was then subjected to high performance liquid chromatography using Q-Sepharose® Fast Flow (Pharmacia, Uppsala, Sweden) for anion exchange. The column was pre-equilibrated with 6 M urea, 25 mM Tris·HCl, 20 mM KCl, 0.1 mM EDTA pH 8.0. Elution of the vWF polypeptide utilized the same buffer except that the concentration of KCl was raised to 250 mM. Polypeptide samples used for further assays were redialyzed against 0.15 M NaCl, 20 mM Hepes, pH 7.4. However, long term storage was best achieved in urea buffer (6 M urea, 25 mM Tris·HCl, 20 mM KCl, 0.1 mM EDTA pH 8.0. Final p7E-vWF polypeptide percent amino acid compositions (by acid hydrolysis) compared closely with values predicted from published sequence information (Bonthron, D. et al. and also Mancuso, D. et al. in Example 1, supra; see also Figure 1).
Example 2 - Characterization of the cysteine-free mutant von Willebrand factor fragment produced by expression plasmid p7E

Urea-solubilized and dialyzed polypeptides extracted from inclusion bodies of cultures containing expression plasmids p7E, p7D and pJD18 were analyzed using polyacrylamide gel electrophoresis (PAGE) and immunoblotting.

Characterization by SDS-Polyacrylamide Gel Electrophoresis

The purity and nature of the expression plasmid extracts, which had been urea-solubilized and then extensively dialyzed, were first analyzed using the denaturing sodium dodecylsulfate-polyacrylamide gel electrophoresis procedure of Weber, K. et al. J. Biol. Chem., 244, 4406-4412 (1969), as modified by Laemli, U.K. Nature, 227, 680-685 (1970) using an acrylamide concentration of 10%. The resultant gels were stained with Coomassie blue and compared.

The extract from expression plasmid p7E contains as the major component, the mutant von Willebrand factor polypeptide which migrates with an apparent molecular weight of approximately 36,000 Daltons. The polypeptide appears as a single band under both reducing conditions (addition of between 10 and 100 mM dithiothreitol "DTT" to the sample for 5 min at 100°C prior to running the gel in a buffer also containing the same DTT concentration) and nonreducing conditions, which result is consistent with the substitution of glycine residues for all of the cysteine residues therein. No vWF polypeptide could be extracted from host cells containing p7D expression plasmids as
expected from the opposite orientation of the vWF cDNA insert.

The cysteine-containing vWF polypeptide expressed by host cells containing pJD18 plasmids, and which contains the wild type amino acid sequence of the 52/48 fragment, (herein represented by a residue 441 to 733 cloned fragment) behaved differently under reducing and nonreducing conditions of electrophoresis. The wild-type sequence expressed from pJD18 forms intermolecular disulfide bridges resulting in large molecular weight aggregates which are unable to enter the 10% acrylamide gels. After reduction (incubation with 100 mM DTT for 5 min at 100°C), the vWF peptide migrates as a single band with a molecular weight of approximately 38,000.

Characterization by Immunoblotting

Polypeptides expressed from p7E, p7D and pJD18 were further characterized by immunoblotting ("Western blotting") according to a standard procedure Burnett et al., A. Anal. Biochem., 112, 195-203, (1981) and as recommended by reagent suppliers. Samples containing approximately 10 μg of protein from the urea-solubilized and dialyzed inclusion body extracts of host cells (containing p7E, p7D and pJD18 plasmids) were subjected to electrophoresis on 10% polyacrylamide gels, Laemli, U.K. Nature, 227, 680-685 (1970), in the presence of 2% concentration of sodium dodecyl sulfate.

The proteins were blotted and immobilized onto a nitrocellulose sheet (Schleicher and Schuell, Keene, NH) and the pattern was then visualized using immunoreactivity.

The secondary antibody (\textsuperscript{125}I-rabbit anti-mouse IgG), labelled by the method of Fraker, P.J. et al. Biochem. Biophys. Res. Commun., 80, 849-857 (1978)), was incubated for 60 minutes at 25°C on the nitrocellulose sheet. After rinsing, the sheet was developed by autoradiography.

Peptide extracts from host cells containing p7E and pJD18 expression plasmids display strong immunoreactivity for RG-46 antibody and a weaker but definite affinity for NMC-4 antibody. As expected, peptide extracts from p7D plasmids show no immunoreactivity with either RG-46 or NMC-4.

**Example 3**

Inhibition of botrocetin-induced binding of vWF to platelets by the cysteine-free mutant polypeptide expressed by p7E

It has been demonstrated that botrocetin, extracted from the venom of Bothrops jararaca modulates the \textit{in vitro} binding of multimeric von Willebrand factor to platelets (Read, et al. Proc. Natl. Acad. Sci., 75, 4514-4518 (1978)) and that botrocetin binds to vWF within the region thereof containing amino acid sequence positions 441-733 (of the mature subunit), and
thus the GPIb binding domain. (Andrews, R.K. et al., Biochemistry, 28, 8317-8326 (1989)).

The urea-solubilized and dialyzed polypeptide extracts, obtained (according to the method of Example 1) from cultures containing expression plasmids p7E, p7D and pJD18, were tested without further purification for their ability to inhibit botrocetin-induced vWF binding to formalin-fixed platelets on a dose dependent basis.

Formalin-fixed platelets, prepared according to the method of MacFarlane, D. et al., Thromb. Diath. Haemorrh. 34, 306-308 (1975), were pre-incubated at room temperature for 15 minutes with specified dilutions of peptide extracts obtained from cultures containing pJD18, p7D, and p7E plasmids. Botrocetin, (Sigma, St. Louis, MO) to a final concentration of 0.4 μg/ml, and 125I-labelled multimeric vWF (isolated from human plasma cryoprecipitate according to the method of Fulcher, C.A. et al. Proc. Natl. Acad. Sci. USA, 79, 1648-1652 (1982), and labelled according to the method of Fraker, P.J. et al. Biochem. Biophys. Res. Commun., 80, 849-857 (1978)) were then added to the incubation mixture, and the amount of 125I- vWF bound to the platelets was determined.

125I-vWF binding to the platelets was referenced against 100% binding which was defined as the amount of 125I- vWF bound in the absence of added peptide extracts.

Figure 2 demonstrates that peptide extracts from expression plasmids p7D, and pJD18 (unreduced and
unalkylated) cannot compete with plasma-derived vWF for platelet GPIIb receptor binding sites. The peptide extract from plasmid p7E was effective in a dose dependent manner (using a range of 0 to 100 \( \mu \text{g} \) extract/ml) in inhibiting vWF binding. The concentration of urea-solubilized polypeptide extract (\( \mu \text{g/ml} \)) in the incubation mixture reflects the total protein concentration from the extract. Addition of peptide extracts to the reaction mixture causes certain nonspecific effects which raise apparent initial binding to 110% of the value found in the absence of the added peptide extracts. The \(^{125}\text{I-vWF} \) concentration used was 2\( \mu \text{g/ml} \).

**Example 4**

Expression of a mutant vWF fragment of reduced cysteine content containing a disulfide-dependant conformation

Utilizing the procedures of Example 1, except as modified below, a mutant vWF polypeptide fragment (corresponding to the mature vWF subunit sequence from residue 441 to residue 733) was prepared in which the cysteines at positions 459, 462, 464, 471 and 474 were each replaced by a glycine residue. Cysteine residues were retained at positions 509 and 695, and allowed to form an intrachain disulfide bond.

Site directed mutagenesis was performed only with oligonucleotides No. 459 and 471, thereby substituting glycine codons only at positions 459, 462, 464, 471 and 474. Upon completion of mutagenesis procedures, the sequence of the mutant vWF cDNA was confirmed using the single-stranded dideoxy method.
The double-stranded form of the vWF cDNA insert (containing 5 cysteine to glycine mutations) was then removed from M13mp18 phage by treatment with EcoRI and HindIII restriction endonucleases, modified as in Example 1 with BamHI linkers, and cloned into pET-3A. The pET-3A vehicle so formed is referred to as "p5E" or p5E expression plasmid.

The p5E expression plasmids were then cloned into ampicillin sensitive *E. coli* strain BL21(DE3), Novagen Co., Madison, WI, according to the procedure of Hanahan, D., *J. Mol. Biol.*, 166, 557-580 (1983). The p5E mutant polypeptide was expressed from cultures of *E. coli* BL21(DE3) following the procedure of Example 1 except that solubilization of inclusion body pellet material in the presence of 8 Molar urea need not be continued beyond the initial 2 hour period at room temperature, at which point redissolved material had reached a concentration of 200 µg/ml. Oxidation of cysteine residues 509 and 695 to form a disulfide bond was accomplished by dialysis overnight against Hepes-buffered saline. Formation of intrachain rather than interchain disulfide bonds is favored by allowing thiol oxidation to proceed at a low protein concentration such as 50-100 µg/ml.

As in Example 1 pertaining to the p7E extracts, final purification of urea-solubilized inclusion body preparations was accomplished by dialysis against the 6 M guanidine and 6 M urea buffers followed by anion exchange chromatography.
Example 5 - Characterization of the mutant vWF fragment produced by expression plasmid p5E

The mutant von Willebrand factor polypeptides produced by cultures containing expression plasmid p5E were characterized utilizing the procedures of Example 2, and in particular compared with the vWF fragment expressed by plasmid p7E.

Urea-solubilized and dialyzed polypeptides extracted from inclusion bodies (according to the procedure of Example 4) were compared with similar extracts from p7E plasmid cultures produced as in Example 1.

Characterization by SDS-Polyacrylamide Gel Electrophoresis

The denaturing sodium dodecylsulfate gel procedure of Example 2 was used to compare the p5E vWF fragments, which can form disulfide bonds using cysteine residues 509 and 695, with the p7E fragment which has no cysteine residues. Electrophoresis was conducted using 7.5 μg of protein extract per lane on 10% acrylamide gels under reducing (100 mM dithiothreitol) and non-reducing conditions.

Under reducing conditions, and after staining with Coomassie blue, extracts from p7E and p5E have identical electrophoretic mobilities.

Electrophoresis under nonreducing conditions, however, demonstrates the effects of disulfide bonds involving residues 509 and 695. A substantial amount
of the p5E extract appears as a high molecular weight complex (resulting from interchain disulfide bonds) which enters the gel only slightly. Densitometric scanning of the gels of initial preparations indicates that approximately 25% of the p5E polypeptide material found on nonreducing gels is represented by monomers of the 441-733 fragment having an apparent molecular weight of approximately 38,000. The percent of monomer present in p5E extracts can be improved significantly by conducting urea solubilization, dialysis, and thiol oxidation at a more dilute protein concentration, such as 50-100 µg/ml, to favor intrachain rather than interchain disulfide bond formation.

This p5E monomeric species has a slightly higher mobility during electrophoresis under nonreducing conditions than the comparable p7E product species which has no cysteine residues. The mobilities of these p5E and p7E monomeric 38 kDa species appear identical under reducing conditions. The slightly accelerated mobility of a polypeptide which retains tertiary structure in the presence of SDS under nonreducing conditions, when compared to the mobility of the homologous polypeptide which the anionic detergent converts completely into a negatively charged fully rigid rod under said conditions, is generally considered suggestive of the presence of an intrachain disulfide bond.

Characterization by Immunoblotting

The behavior of p5E and p7E extracts were also examined using immunological methods.
As in Example 2, vWF-specific murine monoclonal antibodies RG-46 and NMC-4 were used as probes. RG-46 has been demonstrated to recognize as its epitope a linear sequence of amino acids, comprising residues 694 to 708, within the mature von Willebrand factor subunit. The binding of this antibody to its determinant is essentially conformation independent. Mohri, H. et al., *J. Biol. Chem.*, 263(34), 17901-17904 (1988).

NMC-4 however, has as its epitope the domain of the von Willebrand factor subunit which contains the glycoprotein Ib binding site. Mapping of the epitope has demonstrated that it is contained within two discontinuous domains (comprising approximately mature vWF subunit residues 474 to 488 and also approximately residues 694 to 708) brought into disulfide-dependent association, Mohri, H. et al., supra, although it was unknown whether the disulfide bond conferring this tertiary conformation in the native vWF molecule was intrachain or interchain. Id. at 17903.

7.5 µg samples (of protein) were first run on 10% SDS polyacrylamide gels so that the antigenic behavior of particular bands (under reducing and nonreducing conditions) could be compared with results obtained above by Coomassie blue staining. Immunoblotting was performed as in Example 2 to compare p5E and p7E extracts.

Application of antibody to the nitrocellulose sheets was usually accomplished with antibody solutions prepared as follows. Mice were injected with B-lymphocyte hybridomas producing NMC-4 or RG-46.
Ascites fluid from peritoneal tumors was collected and typically contained approximately 5 mg/ml of monoclonal antibody. The ascites fluid was mixed (1 part per 1000) into blocking fluid (PBS containing 5% (w/v) non-fat dry milk, Carnation) to minimize non-specific background binding. The antibody-containing blocking fluid was then applied to the nitrocellulose.

Under nonreducing conditions, the single chain p5E polypeptide fragment (representing the sequence from residue 441 to residue 733) displayed an approximate 120 fold increase in binding affinity for NMC-4 compared to the comparable cystein-free species isolated from p7E also representing the primary sequence from residue 441 to 733. After electrophoresis under reducing conditions (utilizing 100 mM DTT), the single chain p5E species showed a remarkably decreased affinity for NMC-4, which was then very similar to that of the cysteine-free p7E species under either reduced or nonreduced conditions. NMC-4 also fails, under reducing or non-reducing conditions, to recognize as an epitope disulfide-linked dimers from the p5E extract.

The nitrocellulose filters used to produce autoradiographs based on NMC-4 were rescreened with RG-46 by subtracting the initial NMC-4 exposure response, which was kept low through a combination of low antibody titer and short exposure time. The binding of RG-46 to the p7E 36,000 kDa polypeptide on the filters is the same whether reducing or non-reducing conditions were chosen, consistent with the replacement of all cysteines by glycine in the expressed polypeptide.
A large molecular weight vWF antigen (reactive to RG-46) is present in the p5E polypeptide extract under nonreducing conditions. These p5E vWF aggregates (reflecting interchain disulfide bonds) migrate under reducing conditions in the same position as the p7E polypeptide indicating disruption of their disulfide contacts. However, the large p5E interchain disulfide aggregates which are readily recognized under nonreducing conditions by RG-46 are not recognized by NMC-4 under either reducing or nonreducing conditions. It is thus demonstrated that the disulfide bond between residues 509 and 695 in native multimeric vWF subunits represents an intrachain contact.

Example 6 - Inhibition of the binding of an anti-GPIb monoclonal antibody by p5E polypeptide


To assess the inhibitory activity of p5E extracts on antibody binding, a concentration of LJ-Ib1 was first selected which would, in the absence of p5E extracts, provide half-maximal binding.

LJ-Ib1 was iodinated by the procedure of Fraker, D.J. et al., Biochem. Biophys. Res. Commun., 80, 849-857 (1978) using I\textsuperscript{125} from Amersham, Arlington Heights, IL and Iodogen (Pierce Chemical Co., Rockford, IL).
Washed platelets were prepared by the albumin density gradient technique of Walsh, et al., *Br. J. Haematol.*, 36, 281-298 (1977), and used at a count of 1 x 10^8/ml. Half-maximal binding of antibody to platelets was observed at 10 μg/ml LJ-Ib1 concentration, which concentration was selected for p5E polypeptide inhibition studies.

The p5E polypeptide extract was purified according to the procedure of Example 4 including final purification of the urea-solubilized inclusion body preparation by dialysis against 6.0 M guanidine and urea solutions followed by Q-Sepharose® chromatography.

To evaluate binding, platelets were incubated for 30 minutes at 22-25°C with LJ-Ib1 (10 μg/ml) and concentrations of purified p5E protein (0.002-10.0 μMolar) as indicated in Figure 3. Inhibition was plotted in the presence of 2 μg/ml botrocetin, Sigma Chemical Co., St. Louis, MO, (Figure 3, dark circles) and in the absence of botrocetin (open circles).

Less than 5 percent of the ^125^I label bound to the platelets was contributed by labelled substances other than LJ-Ib1 as determined by binding competition experiments in the presence of a 100 fold excess of unlabelled LJ-Ib1. Background labelling was subtracted from data points. Binding of ^125^I LJ-Ib1 was expressed as a percentage of a control assay lacking recombinant polypeptides. Fifty percent inhibition of ^125^I LJ-Ib1 binding to platelets was achieved at 10 μM of p5E polypeptide without botrocetin whereas in the presence of botrocetin (2 μg/ml), 50% inhibition may be achieved at less than 0.1 μM. It is known that botrocetin
induces in circulating multimeric von Willebrand factor and single subunits thereof a conformational change which enhances or permits binding to the GPIIbα receptor. This example demonstrates that the p5E polypeptide (containing an intrachain cysteine 509-695 bond) behaves very much like native circulating von Willebrand factor with respect to how its activity is modulated by botrocetin. Structural similarity is therefore indicated.

Example 7 — Expression of homodimeric 116 kDa von Willebrand factor fragment in stable mammalian transformants

This example is illustrative of conditions under which a DNA sequence encoding the mature vWF subunit fragment having an amino terminus at residue 441 (arginine) and a carboxy terminus at residue 730 (asparagine) may be expressed, and of the secretion from cultured mammalian host cells of a glycosylated homodimeric form of the 441-730 vWF fragment having native tertiary structure.

Expression of the 116 kDa homodimer is achieved using a DNA construct in which the following structural elements are assembled in a 5' to 3' direction (referring to the coding or nontranscribed strand):

(A) a eucaryotic consensus translation initiation sequence, CCACC; and

(B) the initiating vWF methionine codon followed by the remaining 21 amino acids of the vWF signal peptide; and

(C) the coding sequence corresponding to the first three amino acids from the amino terminus region of the vWF propeptide; and
(D) the coding sequence for vWF amino acid residues 441-730; and
(E) the "TGA" translation termination codon.

Preparation of a cDNA Clone from Pre-pro-von Willebrand Factor mRNA

The cDNA clone, pvWF, encoding the entire pre-pro-vWF gene was obtained from Dr. Dennis Lynch, Dana-Farber Cancer Institute, Boston, MA and was prepared as described in Lynch, D.C. et al., Cell, 41, 49-56 (1985). Preparation of pvWF was described in Example 1.

Primer Directed Amplification of cDNA - Phase I

The cDNA representing the full length pre-pre-vWF gene from pSP64 was subjected to enzymatic amplification in a polymerase chain reaction according to the method of Saiki, R.K. et al. Science, 239, 487-491 (1988), as described in Example 1.

For PCR amplification, the following oligonucleotides were synthesized by the phosphoramidite method, Sinha, et al., Tetrahedron Letters, 24, 5843 (1983), using a model 380B automated system, Applied Biosystems, Foster City, CA.

Oligonucleotide (7) - see SEQ ID NO: 8

\[ 5' - \text{GTGCAGGCCACCATGATTCTTGCCAGA} - 3' \]

SalI Met

Oligonucleotide (8) - see SEQ ID NO: 9

\[ 5' - \text{TCAGTTTCTAGATACAGCCC} - 3' \]

XbaI
In designing the oligonucleotides used herein, reference was made to the established nucleotide sequence of the pre pro-vWF gene, Bonthon, D. et al., *Nucl. Acids Res.*, 14(17), 7125-7127 (1986); Mancuso, D. et al., *J. Biol. Chem.*, 264(33), 19514-19527 (1989).

Oligonucleotide (7) was used to create a SalI restriction site fused 5' to a eucaryotic consensus translation initiation sequence (CCACC) preceding the initiating methionine codon of the vWF cDNA. See Kozak, M. *Cell*, 44, 183-292 (1986).

Oligonucleotide (8) hybridizes with the non-transcribed strand (coding strand) of the vWF cDNA and overlaps with nucleotides which are approximately 360 base pairs from the initiating methionine in the pre-pro-vWF cDNA, thus spanning (at residues 120 and 121 within the pre-pro-vWF cDNA sequence) an XbaI restriction site.

The polymerase chain reaction therefore synthesized a cDNA fragment, containing (reading from 5’ to 3’ on the coding strand) a SalI site, a consensus initiation sequence, an initiating methionine codon, the codon sequence for the signal peptide, and approximately, the first 100 codons of the propeptide, followed by an XbaI site.

**Insertion of cDNA into M13mp18 Cloning Vehicle**

The amplified cDNA fragment was then inserted, using SalI and XbaI restriction enzymes, into the double stranded replicative form of bacteriophage M13mp18 which contains a multiple cloning site having

Primer Directed Amplification of cDNA - Phase II

cDNA corresponding to mature vWF amino acid residues 441 to 732 was then amplified in a polymerase chain reaction. For amplification, the pvWF clone encoding the entire pre-pro-vWF gene was used. Alternatively, a cDNA corresponding to mature subunit residues 441 to 732 may be prepared and then amplified directly from platelet mRNA following the procedure of Newman, P.J. et al. J. Clin. Invest., 82, 739-743 (1988).

Suitable flanking oligonucleotides were synthesized as follows:

Oligonucleotide (9) - see SEQ ID NO: 10

\[ 5' - \text{AC·GAATTC·CGG·CGT·TTT·GCC·TCA·GGA} - 3' \]
\[ \text{EcoRI Arg}_{441}\text{Arg}_{442} \]

Oligonucleotide (10) - see SEQ ID NO: 11

\[ 5' - \text{G·AAGCTT·AC·CAT·GGA·GTT·CCT·CTT·GGG} - 3' \]
\[ \text{HindIII Met Ser Asn Arg Lys Pro 732 731 730 729 728 727} \]
\[ \text{or} \]
The ends of the double stranded vWF cDNA fragment product were then modified with BamHI linkers (Roberts, R.J. et al. Nature, 265, 82-84 (1977)), digested with BamHI, and inserted into the BamHI site of pAD1, which site is directly downstream(3') from the XbaI site. The resultant plasmid was designated pAD2.

Loopout Mutagenesis of pAD2.

Site-directed (loopout) mutagenesis was then performed to synchronize the reading frames of the first insert with the second insert simultaneously deleting all propeptide codon sequence (except that encoding the first 3 amino terminal residues of the propeptide), and the remaining bases between the XbaI and BamHI sites.

As a loopout primer, the following oligonucleotide was utilized which encodes the four carboxy-terminal amino acid residues of the signal peptide, the three amino-terminal residues of the propeptide, and amino acid residues 441 to 446 of the mature vWF subunit sequence.

Oligonucleotide (11) – see SEQ ID NO: 12

5' - GGGACCCTTTTGCGAGAAGGACGCGTTTTGCGCTCAGGA - 3'  
\[ \text{Arg}_{441} \text{ Gly}_{446} \]

The loopout of undesired nucleotide sequence was accomplished following the procedure of Kunkel, T.A., Proc. Natl. Acad. Sci. USA, 82, 488-492 (1985). This procedure involves the performance of a series of steps...
to take advantage of conditions which select against a uracil containing DNA template:

(A) M13mp18 phage (containing cDNA corresponding to the consensus translation initiation sequence, the signal peptide, approximately the first 121 amino acids of the propeptide, residual intervening M13mp18 polylinker sequence, and codons corresponding to mature subunit sequence residues 441 to 732) is grown in an E. coli CJ236 mutant dut ung strain in a uridine rich medium. Since this E. coli strain is deficient in deoxyuridine triphosphatase (dut-), an intracellular pool of dUTP accumulates which competes with dTTP for incorporation into DNA. (see Shlomai, J. et al. J. Biol. Chem., 253(9), 3305-3312 (1978). Viral DNA synthesized under these conditions includes several uracil insertions per viral genome and is stable only in an E. coli strain which is incapable of removing uracil, such as (ung-) strains which lack uracil glycosylase. Uracil-containing nucleotides are lethal in single stranded (+) M13mp18 DNA in ung+ strains due to the creation of abasic sites by uracil glycosylase.

(B) Single-stranded (+) viral DNA is isolated from culture media in which phage were grown in E. coli strain CJ236 dut ung-. The single stranded (+) form of the virus contains the specified vWF cDNA at its multiple cloning site. This cDNA is equivalent to the transcribed vWF cDNA strand.
(C) Oligonucleotide (11) is then annealed in vitro to single stranded (+) phage DNA, thereby looping out the undesired sequence. Generally, a wide range of oligonucleotide concentrations is suitable in this procedure. Typically 40 ng of oligonucleotide was annealed to 0.5-1.0 µg M13mp18 phage (+) DNA.

(D) All missing sequence of the M13mp18(−) strand is then completed in vitro using T7 DNA polymerase and T4 DNA ligase in an environment containing dTTP, dGTP, dATP and dCTP, thereby generating a chimeric VWF cDNA sequence without the undesired intermediate sequence.

(E) The double stranded M13mp18 phage, now containing a thymine normal (−) strand and a (+) strand with several uracil substitutions, is transformed into a wild type E.coli XL-1 Blue (Stratagene, La Jolla, CA) strain which contains normal levels of uracil glycosylase and deoxyuridine triphosphatase.

(F) Uracil glycosylase and other enzymes present in the new host initiate destruction of the uracil-containing (+) strand of the double stranded phages, leading after replication in the host of remaining phage (−) strand DNA to the presence of stable thymine-normal double stranded (RF) DNA which reflects the desired deletion. Upon completion of mutagenesis procedures, the sequence of the VWF cDNA insert was confirmed using the single
stranded DNA dideoxy method. (Sanger, F. et al., supra).

A second mutagenesis procedure, following steps (A) to (F) above, was performed to add to the cDNA insert a translation termination codon (TGA), and an XbaI restriction site (TCTAGA). The oligonucleotide, again synthesized by the phosphoramidite method and containing also sequence homology at its 3’ end with the M13mp18 vehicle sequence, was as follows. The stop codon was added after residue 730.

Oligonucleotide (12) - see SEQ ID NO: 13

5’- GGGCCCAAG•AGG•AAC•TGA•TCTAGA•AAGCTTGCCACTGCG -3’
Arg<sub>729</sub>Asn<sub>730</sub> XbaI

The final M13mp18 recombinant containing the desired construct as a SalI - XbaI insert was designated pAD3-1. In addition to the XbaI site created 3’ to the termination codon, an XbaI site exists in the polylinker region of M13mp18 directly 5’ to the SalI site. The vWF insert was again sequenced by the dideoxy method to verify organization and integrity of the components.

Cloning of the SalI – XbaI Fragment of pAD3-1 Into the pBluescript II KS(−) Vector

The SalI-XbaI fragment was then removed from pAD3-1 (as contained within the XbaI-XbaI fragment) and inserted into pBluescript II KS(−) vector (Stratagene, La Jolla, CA) which had been previously digested with XbaI. pBluescript II KS(−) contains an XhoI restriction site which is 5’ to the XbaI insert and a NotI site which is directly 3’ to the XbaI insert. A
resultant plasmid selected as having the proper insert orientation was designated pAD3-2.

Alternatively, the SalI-XbaI fragment itself may be removed from pAD3-1 and inserted into pBluescript KS(+) vector which would have been digested previously with SalI and XbaI restriction enzymes. The resultant plasmid, a form of pAD3-2, would also contain an XhoI restriction site which is directly 5' to the SalI site, and a NotI site which is directly 3' to the XbaI site. Such a construct (see below) is also suitable for insertion into pCDM8<sup>neo</sup> vectors.

Construction of Plasmids for Integration into Mammalian Cells

A selection procedure, based on aminoglycosidic antibiotic resistance, was then employed to select continuously for transformants which retained the vWF expression plasmid.

pCDM8 vector (developed by B. Seed et al. Nature, 329, 840-842 (1987) and available from Invitrogen, San Diego, CA) was modified by Dr. Timothy O'Toole, Scripps Clinic and Research Foundation, La Jolla, CA to include a neomycin resistance gene (phosphotransferase II) that was cloned into the BamHI restriction site of pCDM8 as a part of a 2000 base pair BamHI fragment. The site of the BamHI insert is indicated by an arrow in Figure 4. The protein produced by the neomycin(neo) gene also confers resistance against other aminoglycoside antibiotics such as Geneticin® G418 sulfate (Gibco/Life Technologies, Inc., Gaithersburg, MD). The neo gene is provided by the Tn5 transposable element and is widely distributed in procaryots. Lewin, J., Genes, 3rd ed.,

Several other suitable expression vectors containing neomycin resistance markers are commercially available: pcDNA 1™ (Invitrogen, San Diego, CA), Rc/CMV (Invitrogen, San Diego, CA) and pMAP™ (Clontech, Palo Alto, CA). If necessary, the VWF fragment may be differently restricted or modified for expression capability in these other expression plasmids.

The XhoI-NotI fragment of pAD3-2 was therefore inserted into pCDM8™ which had been restricted with XhoI and NotI. Ampicillin sensitive *E. coli* strain XS-127 cells (Invitrogen, San Diego, CA) were transformed with the resultant ligated DNA mixture following the method of Hanahan, D., *J. Mol. Biol.*, 166, 557-580 (1983).

Plasmids from resultant colonies were characterized by restriction mapping and DNA sequencing to identify colonies which contained the intended insert. One such appropriate plasmid (designated pAD5/WT) was maintained in *E. coli* strain XS-127, and was selected for mammalian cell transformation procedures.

Prior to use in transforming mammalian cells, supercoiled plasmids (pAD5/WT) were recovered from host *E. coli* by an alkaline cell lysis procedure, Birnboim, H.C. and Doly, J., *Nucleic Acids Research*, 7,1513 (1979), followed by purification by CsCl/ethidium

**Transformation of Chinese Hamster Ovary Cells**


CHO-K1 cells were grown at 37°C in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco/Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% heat-inactivated fetal calf serum (FCS), 0.5 mM of each nonessential amino acid (from NEAA supplement, Whittaker, Walkersville, MD) and 2 mM L-glutamine under a 5% CO₂ atmosphere, and then subcultured 24 hours prior to transformation at a density of 1.5 x 10⁵ cells per 60 mm tissue culture dish (approximately 25% of confluence). CHO-K1 cells have a doubling time in DMEM/10%FCS of approximately 16 hours under these conditions.

To accomplish transformation, pAD5/WT plasmids were recovered from cultures of *E.coli* strain XS-127, according to the method of Birnboim, H.C. and Doly, J., Nucleic Acids Research, 7, 1513 (1979). Ten µg of plasmids were applied to the cells of each 60 mm dish in a calcium phosphate solution according to the method of Chen et al., supra. After inoculation with plasmid, the cells were maintained in DMEM/10% FCS for 8 hours at 37°C in a 5% CO₂ atmosphere.
The growth medium was then replaced with a solution of phosphate-buffered saline, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7H₂O/1.4 mM KH₂PO₄, pH 7.4, hereinafter "PBS", containing also 10% (v/v) glycerol. The cultures were then maintained in glycerol-PBS for 2 minutes to increase the efficiency of transformation (see Ausubel, et al., eds. Current Protocols in Molecular Biology, p.9.1.3, Wiley & Sons (1987). After 2 minutes the glycerol-PBS solution was replaced with DMEM/10% FCS.

After approximately 24 hours of growth at 37°C in a 5% CO₂ atmosphere, the cells were trypsinized as follows. Growth medium for each dish was replaced by 3 ml of 0.25% trypsin in PBS. Trypsinization was conducted for 3 minutes. The trypsin-containing medium was removed and the dishes were then placed in the incubator for a further 15 minutes after which the cells were resuspended in DMEM containing 10% fetal calf serum. The cells from each dish were then split 20 fold, and plated at a density of 3 x 10⁴ cells/60 mm dish (approximately 5% of confluence).

Production of stable transformants, which have integrated the plasmid DNA, was then accomplished by adding Geneticin® G418 sulfate to the 60 mm dishes to a concentration of 0.8 mg/ml. Growth was continued for 10-14 days at 37°C in a 5% CO₂ atmosphere. Surviving independent colonies were transferred to 12-well plates using cloning rings and then grown for another seven days in DMEM/10% FCS supplemented with 0.8 mg/ml of Geneticin®. Under these conditions, 3 to 7 surviving colonies per plate were apparent after 10-14 days. Approximately 100 stable transformants can be
isolated from each original 60 mM dish originally containing approximately
5 x 10^5 cells at a plate density of 50-70% of confluence.

Fifty to seventy percent of G418-resistant cell lines produce the 441-730 mature vWF subunit fragment. The specific geometry of integration of each clone presumably prevents expression in all cases. Stable transformants were then cultured and maintained at all times in medium containing Geneticin® G418 sulfate (.8 mg/ml) to apply continuous selection.

Colonies expressing the recombinant 441-730 vWF polypeptide were detected by dot-blot analysis on nitrocellulose after lysis in disruption buffer (see Cullen, Methods in Enzymology, 152, 684-704 (1987)) comprising 10 mM Tris·HCl, pH 7.8, 150 mM NaCl, 5 mM EDTA, 10 mM benzamidine, 1 mM PMSF, 1% (w/v) Non-Idet 40 (an octylphenol-ethylene oxide condensate containing an average of 9 moles of ethylene oxide/mole phenol), Sigma, St. Louis, MO.

RG-46 (see Fugimura, Y. et al. J. Biol. Chem., 261(1), 381-385 (1986) and Fulcher, C.A. et al. Proc. Natl. Acad. Sci. USA, 79, 1648-1652 (1982)) was used as the primary antibody. The secondary antibody (^{125}I-rabbit anti-mouse IgG) which had been labelled by the method of Fraker, P.J. et al. Biochem. Biophys. Res. Commun., 80, 849-857 (1978) was incubated for 60 minutes at 25°C on the nitrocellulose sheet. After rinsing, the nitrocellulose was developed by autoradiography to identify those colonies expressing the vWF fragment.
Secretion of the von Willebrand Factor Fragment

Secretion of the 441-730 mature vWF subunit fragment into the culture medium by CHO-K1 cells was confirmed by immunoprecipitation and immunoaffinity chromatography of culture medium.

Confluent transformed CHO-K1 cells were rinsed three times with PBS to remove bovine vWF and then incubated in DMEM without FCS for 16 hours at 37°C in a 5% CO₂ atmosphere. To a 5 ml volume of the culture medium was added a 1/10 volume (0.5 ml) of 10x immunoprecipitation buffer (10xIPB) which comprises 100 mM Tris·HCl, pH 7.5, 1.5 M NaCl, 10 mM EDTA, and 10% (w/v) Non-ident 40. It has been established that bovine vWF-derived polypeptides present in fetal calf serum do not react with NMC-4.

The mixture was then incubated for 16 hours at 4°C with approximately 0.05 mg of NMC-4 or 0.05 mg of RG-46 murine monoclonal anti-vWF antibody (or 0.1 mg of both) allowing formation of IgG-vWF complexes. Immune complexes were precipitated by taking advantage of the affinity of protein A (isolated from the cell wall of Staphylococcus aureus) for constant regions of heavy-chain antibody polypeptides following generally the method of Cullen, B. et al., Meth. Enzymology, 152, 684-704 (1987). See also Harlow, E. et al. eds, Antibodies, A Laboratory Manual, Chapters 14-15, Cold Spring Harbor Laboratory Press (1989).

Protein A-Sepharose® beads were purchased from Sigma, St. Louis, MO. Immune complexes were then precipitated with the beads in the presence of 3 M
NaCl/1.5 M glycine (pH 8.9), and washed twice with 1x IPB and then once with 1x IPB without Non-ident 40.

Immunoprecipitated proteins were then electrophoresed in polyacrylamide gels containing sodium dodecyl sulfate (SDS-PAGE) following the method of Weber, K. et al., J. Biol. Chem., 244, 4406-4412 (1969), or as modified by Laemli, U.K., Nature, 227, 680-685 (1970), using an acrylamide concentration of 10%. Samples of immune-complexed vWF protein were dissociated prior to electrophoresis by heating at 100°C for 5 minutes in non-reducing and 2% SDS-containing acrylamide gel sample buffer to disrupt non-covalent bonds. The protein A-Sepharose®4B beads were spun down and discarded. Visualization was accomplished with Coomassie blue staining which revealed the dominant vWF-derived polypeptide species to have an apparent molecular weight, based on molecular weight markers, of about 116,000 daltons.

Protein bands in duplicate gels were blotted and immobilized onto nitrocellulose sheets (Schleicher & Schuell Co., Keene, NH) and the pattern was then visualized using immunoreactivity according to the highly sensitive "Western blot" technique. Burnette, et al., A. Anal. Biochem., 112, 195-203 (1981).

662-669 (1985)), both of which have epitopes within the expressed vWF polypeptide of this invention.

The secondary antibody (^{125}I-rabbit anti-mouse IgG), labelled by the method of Fraker, P.J. et al., Biochem. Biophys. Res. Commun., 80, 849-857 (1978)), was incubated for 60 minutes at 25°C on the nitrocellulose sheet. After rinsing, the sheet was developed by autoradiography.

Growth medium from non-transformed CHO-K1 cells shows no immunoreactivity with RG-46 and NMC-4 anti-vWF monoclonal antibodies under identical conditions.

The 116 kDa fragment may also be isolated from the culture medium of CHO-K1 cells using immunoaffinity chromatography. Approximately 300μg of the 116 kDa fragment can be recovered from 500 ml of culture medium derived from transformed CHO-K1 culture plates using NMC-4 antibodies coupled to particles of Sepharose^{4B}.

**Example 8 - Induction of platelet aggregation by the homodimeric 116 kDa von Willebrand factor fragment derived from the culture medium of stable CHO-K1 transformants**

The tryptic 116 kDa fragment has been previously characterized as a dimer consisting of two identical disulfide-linked subunits each corresponding to the tryptic 52/48 kDa fragment of vWF and containing the mature subunit sequence from residue 449 to residue 728. Owing to its bivalent character, the dimeric 116 kDa fragment can support ristocetin-induced platelet aggregation whereas the constituent 52/48 kDa subunit
cannot (see Mohri, H. et al., J. Biol. Chem., 264(29), 17361-17367 (1989)).

Stable pAD5/WT CHO-K1 transformants, and untransformed CHO-K1 cells as controls, were each grown to 90% of confluence in DMEM/10% FCS, at 37°C in a 5% CO₂ atmosphere. The 60 mm plates were then rinsed twice with PBS and the incubation was continued in DMEM (without FCS) for 24 hours. The resultant serum-free culture medium was collected and concentrated (at 18°C) 300 fold in a centrifugation-filtration apparatus, Centricon 30, Amicon Co., Lexington, MA.

A dose-dependent platelet aggregation curve results from the addition of concentrated culture medium from pAD5/WT transformed cells to platelets. No aggregation was seen in the presence of control culture medium derived from untransformed CHO-K1 cells. Platelets for the assay were prepared using albumin density gradients according to the procedure of Walsh, et al. British J. of Hematology, 36, 281-298 (1977).

Aggregation was monitored in siliconized glass cuvettes maintained at 37°C with constant stirring (1200 rpm) in a Lumi-aggregometer (Chrono-Log Corp., Havertown, PA). Aggregation experiments followed generally the procedure of Mohri, H. et al., J. Biol. Chem., 264(29), 17361-17367 (1989). Two to ten μl quantities of 300-fold concentrated FCS-free DMEM from cultures of pAD5/WT-transformed and control untransformed CHO-K1 cells (CM) were brought up to 100 μl by dilution with "Hepes" buffered saline, comprising 20 mM Hepes, N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid], (pH 7.4), and 0.15 M NaCl. The 100 μl samples were then mixed with 200 μl of platelet suspension (4 x 10⁹/ml)
and then incubated with stirring in the aggregometer for 5 minutes. Ristocetin was then added to a final concentration of 1mg/ml at the injection timepoints (time zero). Aggregation was monitored by recording changes in light transmittance. Platelet aggregation can be observed with as little as 100 μl of unconcentrated serum-free medium from pAD5/WT-transformed cell lines. Serum-free medium from control untransformed cultures concentrated up to 300 fold, and assayed at up to 10 μl concentrated medium/100 μl sample did not induce platelet aggregation.

Preincubation with Monoclonal Antibodies

As a further control to confirm the specificity of the ristocetin-induced 116 kDa vWF fragment-platelet interaction, platelets were preincubated with anti-platelet glycoprotein Ib monoclonal antibody LJ-Ib1 which has been specifically demonstrated to block vWF-platelet GPIb-IX receptor interaction (Handa, et al., J. Biol. Chem., 261, 12579-12585 (1986)).

The effect of preincubating the platelets with platelet surface receptor-specific LJ-Ib1 monoclonal antibodies prior to conducting the aggregation assay was examined. Platelets subjected to this preincubation did not exhibit an aggregation response whereas platelets similarly preincubated with monoclonal antibody LJ-CP3 (Trapani-Lombardo et al., J. Clin. Invest., 76, 1950-1958 (1985) gave an effective aggregation response. LJ-CP3 has been demonstrated to block platelet GPIIb/IIIa receptor sites and not vWF-specific GPIb-IX receptors. To perform the assays antibody LJ-Ib1 or antibody LJ-CP3 was added, at a
concentration of 100 µg/ml, to the platelet/serum mixture while the mixture was being stirred in the aggregometer, and at a timepoint one minute prior to the point when ristocetin (to 1 mg/ml) was added. The assays were otherwise identical to those described above. Changes in light transmittance were monitored for an approximate 5 minute (LJ-Ib1) or 4 minute (LJ-CP3) interval.

Example 9 - Construction of a mammalian transformant for the expression of the monomeric 441-730 mature von Willebrand factor subunit fragment with cysteine-to-glycine mutations at residues 459, 462 and 464

This example is illustrative of conditions under which a DNA sequence encoding a mature vWF subunit fragment, which has an amino terminus at residue 441 (arginine) and a carboxy terminus at residue 730 (asparagine) and which further contains glycine residues substituted for cysteine residues at positions 459, 462 and 464 thereof, can be constructed and transfected into mammalian cells.

The SalI-XbaI insert of pAD3-2 (see Example 7) was removed by restriction and then cloned into pcDNA1 vector (Invitrogen, San Diego, CA) which had been previously digested with XhoI and XbaI restriction enzymes. Since XhoI and SalI restriction sites contain identical internal sequences -TCGA- / -AGCT- , a SalI restricted fragment may be annealed into an XhoI site. The fragments were ligated with T4 DNA ligase; however the integrity of the XhoI site was not restored. This plasmid construct was designated pAD4/WT.
Site-directed mutagenesis using M13mp18

pAD4/WT was restricted with EcoRI and SmaI enzymes. pcDNA1 vector contains an EcoRI site within its polylinker region which is upstream from the XhoI ("SalI") site but contains no SmaI site. As shown in Figure 1 (SEQ ID NO: 1), a unique SmaI site (CCCGGG) is contained within the vWF cDNA insert, spanning mature subunit residues 716 (glycine) to residue 718 (glycine).

Accordingly, an approximate 950 base pair EcoRI-SmaI fragment of pAD4/WT was subcloned into the EcoRI-SmaI site within the polylinker region of M13mp18 phage. The vWF sequence in M13mp18 was then mutagenized and reinserted into the previously restricted pAD4/WT construct leading to reassembly of the intact residue 441-730 vWF sequence.

The mutagenesis followed the procedure of Example 1 and Kunkel, T.A., supra, and utilized the following oligonucleotide.

Oligonucleotide (13) - see SEQ ID NO: 14

3' - GGA<sub>3</sub>C<sub>4</sub>T<sub>5</sub>G<sub>6</sub>C<sub>7</sub>C<sub>8</sub>G<sub>9</sub>G<sub>10</sub>T<sub>11</sub>C<sub>12</sub>A<sub>13</sub>C<sub>14</sub>G<sub>15</sub>G<sub>16</sub>C<sub>17</sub>CT<sub>18</sub>A<sub>19</sub>A<sub>20</sub>C<sub>21</sub>G<sub>22</sub>G<sub>23</sub>G<sub>24</sub>T<sub>25</sub>5'  

5' - cctgagccagggccagattggcaggtgttggct - 3'  

Gly<sub>459</sub> Gly<sub>462</sub> Gly<sub>464</sub>

The hybridizing oligonucleotide is shown (3' → 5') in capital letters and is equivalent to transcribed strand (non-coding strand DNA). Underlined letters indicate the single base mutations for the mutant codons. The equivalent coding strand is shown in lower case letters with the corresponding glycine substitutions identified by three letter designation.
The mutant 950 base pair EcoRI-SmaI fragment was then re-inserted into the EcoRI-SmaI site of the previously restricted pAD4/WT plasmid. The mutant construct was designated pAD4/Δ3C. To facilitate long-term storage and propagation, pAD4/Δ3C was transformed into ampicillin sensitive *E. coli* strain XS-127 according to the method of Hanahan, D., *J. Mol. Biol.*, 166, 557-580 (1983).

Consistent with the procedures of Example 1, the sequence of the mutant cDNA was confirmed by the dideoxy method and the plasmid was purified by CsCl/ethidium bromide equilibrium centrifugation.

**Transformation of COS-1 cells**


COS-1 cells were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) (Gibco/Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal calf serum (FCS) under a 5% CO₂ atmosphere, and then subcultured 24 hours prior to transformation at a density of 1.5 x 10⁵ cells/60 mm tissue culture dish (approximately 25% of confluence). COS-1 cells have a doubling time in DMEM/10% FCS of approximately 20 hours under these conditions.

To accomplish transformation, pAD4/Δ3C plasmids were recovered from cultures of *E. coli* strain XS-127.
according to the method of Birnboim, H.C. and Doly, J., Nucleic Acids Research, 7, 1513 (1979). Ten µg of plasmids were applied to the cells of each 60 mm dish in a calcium phosphate solution according to the method of Chen et al., supra. After inoculation with plasmid, the cells were maintained in DMEM/10% FCS for 8 hours at 37°C in a 5% CO₂ atmosphere.

The growth medium was then replaced with a solution of phosphate-buffered saline/10% (v/v) glycerol. The cultures were then maintained in glycerol-PBS for 2 minutes to facilitate the production of transformants (Ausukel, et al. eds, Current Protocols in Molecular Biology, p.9.1.3, Wiley & Sons (1987)). After 2 minutes, the glycerol-PBS solution was replaced with DMEM/10% FCS. Antibiotic resistance was not used to select for stable transformants. The cells were then maintained at 37°C in DMEM/10% FCS in a 5% CO₂ atmosphere.

Example 10 - Transformation of COS-1 cells by pAD4/WT plasmids

COS-1 cells were also transformed successfully with pAD4/WT plasmids. Although antibiotic resistance was not used to select for stable transformants, transient expression of the 116 kDa fragment therefrom was particularly useful for the purpose of comparing the properties of the 116 kDa mutagenized polypeptide produced by pAD4/Δ3C plasmids to those of the pAD4/WT 116 kDa homodimer.

Following the procedures of Example 9, pAD4/WT plasmids were recovered from storage cultures of E.coli strain XS-127. Transformation of COS-1 cells with pAD4/WT was then accomplished using the procedures of
Example 9. The cells were then maintained at 37°C in DMEM/10% FCS in a 5% CO₂ atmosphere.

Example 11 - Construction of mammalian transformants which express mutant 441-730 mature von Willebrand factor subunit fragments wherein each mutant contains a single cysteine-to-glycine substitution

Following the procedures of Example 9, and using suitable oligonucleotides for site-directed mutagenesis, three plasmids (pAD4/G⁴⁵⁹, pAD4/G⁴⁶² and pAD4/G⁴⁶⁴, collectively referred to as "pAD4/Δ1C plasmids") were constructed. Such plasmids are identical to pAD4/WT except that each contains a single base pair mutation which corresponds to a single cysteine to glycine substitution at mature vWF subunit residue positions 459, 462 and 464 respectively. The oligonucleotides used are identical to oligonucleotide (13) used to prepare pAD4/Δ3C except that each contains only one of the three mutant codons of that oligonucleotide, the other two codons being represented by the wild type coding sequence. To facilitate long-term storage and propagation, samples of pAD4/G⁴⁵⁹, pAD4/G⁴⁶², and pAD4/G⁴⁶⁴ were each cloned into ampicillin sensitive E.coli strain XS-127 following the method of Example 9.

Consistent with the procedures of Example 9, the sequences of the mutant cDNAs were confirmed by the dideoxy method and the plasmids were purified by CsCl/ethidium bromide equilibrium centrifugation.

Transformation of COS-1 cells with either pAD4/G⁴⁵⁹, pAD4/G⁴⁶² or pAD4/G⁴⁶⁴ plasmids was accomplished
according to the protocol of Example 9. Antibiotic resistance was not used to select for stable transformants. The cells were then maintained at 37°C in DMEM/10% FCS in a 5% CO₂ atmosphere.

Example 12 - Expression and characterization of von Willebrand factor subunit fragments by COS-1 cells transformed with pAD4/WT and pAD4/Δ3C plasmids

COS-1 cells which had been transformed with pAD4/Δ3C or pAD4/WT plasmids according to the procedures of Examples 9 and 10 respectively were cultured to express the encoded vWF DNA as explained below. COS-1 cells similarly transformed with pcDNA1 plasmid vector (not containing a vWF cDNA insert) were used as controls.

COS-1 cells at a density of 4-5 x 10⁵/60 mm dish were transformed by adding, at time zero, 10 μg of pAD4/WT, pAD4/Δ3C or pcDNA1 plasmid. Following the procedure of Examples 9 and 10, the cells were glycerol-shocked after a period of 8 hours. The cells were then covered with DMEM/10% FCS at 37°C in a 5% CO₂ atmosphere for 32 hours.

The cells for each culture were then rinsed three times with PBS and the incubation was continued with DMEM (without FCS) which was supplemented with ³⁵S-methionine (Amersham Co., Arlington Heights, IL) having a specific activity of 1000 Ci/mmol to a final concentration of 100 μCi/ml. The cells were returned to the incubator for 16 hours, after which time the respective culture media were harvested for
purification by immunoprecipitation of secreted vWF polypeptides.

Immunoprecipitation followed generally the procedure of Example 7. Five ml volumes of culture media were incubated with 0.5 ml of 10X immunoprecipitation buffer, 0.05 mg of NMC-4 antibody and 0.05 mg of RG-46 antibody for 16 hours.

Treatment with protein A-Sepharose®4B was performed according to Example 7. Samples of IgG-complexed vWF protein were dissociated prior to SDS-PAGE in SDS-containing sample buffer.

For analysis of the vWF polypeptides under reducing conditions, the sample buffer was modified to contain 100 mM dithiothreitol (DTT).

Results

The gels were run under reducing and non-reducing conditions and were dried and subject to autoradiography to develop the 35S label. No 35S-labelled protein was detected as an immunoprecipitate derived from control cultures of COS-1 cells (transformed by unmodified pcDNA1 vehicle) under either reducing or non-reducing conditions (see gel lanes designated MOCK).

COS-1 cells transformed with pAD4/WT plasmids produce, under non-reducing conditions, a prominent 35S-labelled band of an approximate apparent molecular weight of 116,000. This value is consistent with proper mammalian glycosylation of the 441-730 fragment.
When run under reducing conditions, no 116 kDa material is apparent, consistent with the reduction of the disulfide bonds which stabilize the 116 kDa homodimer. Under reducing conditions, a prominent $^{35}$S-labelled band is visualized of approximately 52,000 apparent molecular weight. The apparent 52 kDa value is again consistent with proper glycosylation of the reduced monomeric 441-730 fragment.

The gel lanes corresponding to transformation with pAD4/${\Delta}3C$ show no apparent 116 kDa material. Instead a band is apparent, under reducing and non-reducing conditions, at an apparent molecular weight of approximately 52,000.

Thus, mutagenesis to replace cysteine residues 459, 462 and 464 within the 441-730 vWF fragment with glycine residues results in the successful expression of a non-dimerizing polypeptide presumably having only intrachain (471 to 474 and 509 to 695) disulfide bonds. Interaction with NMC-4 (see also Example 7) is known to require an intact 509 to 695 intrachain disulfide bond, thereby demonstrating the presence of native wild type tertiary structure in the polypeptide produced by pAD4/${\Delta}3C$.

The gels also demonstrated the presence of low molecular weight $^{35}$S-labelled material (under reducing and non-reducing conditions) probably indicating that not all vWF polypeptides produced by pAD4/WT constructs successfully dimerize and that proteolysis and/or incomplete glycosylation of the polypeptide may prevent higher yields. Proteolysis and/or incomplete glycosylation also presumably affect the yield of the
monomeric vWF polypeptide produced by the pAD4/Δ3C transformants. Some high molecular weight aggregate material (essentially not entering the gels) is present in non-reduced samples from pAD4/WT and pAD4/Δ3C.

Example 13 - Use of NMC-4 monoclonal antibody to immunoprecipitate vWF polypeptides secreted by pAD4/WT and pAD4/Δ3C transformed COS-1 cells

The NMC-4 monoclonal antibody has as its epitope the domain of the von Willebrand factor subunit which contains the glycoprotein Ib binding site. Mapping of the epitope has demonstrated that it is contained within two discontinuous domains (comprising approximately mature vWF subunit residues 474 to 488 and also approximately residues 694 to 708) brought into disulfide-dependent association by an intrachain (residues 509 to 695) disulfide bond.

Thus, reactivity with NMC-4 is important evidence of whether a particular recombinant 441-730 mature vWF subunit fragment has assumed the tertiary structure of the analogous wild type residue 441-730 domain.

Accordingly, the procedure of Example 12 was followed to characterize vWF polypeptides secreted by pAD4/WT and pAD4/Δ3C transformed COS-1 cells, with the modification that immunoprecipitation of the culture media was effected solely with NMC-4 antibody (0.05 mg NMC-4 per 5 ml of culture media to which 0.5 ml of 10X immunoprecipitation buffer had been added).

Samples were run under reducing and non-reducing conditions. Consistent with the results of Example 12,
the major component isolated from pAD4/WT culture medium has an apparent molecular weight of 116 kDa under non-reducing conditions and 52 kDa under reducing conditions.

Although only a small fraction of the total pAD4/Δ3C derived vWF polypeptide material binds to NMC-4 (compared to conformation independent RG-46), a band of apparent molecular weight of 52 kDa is visible under reducing and non-reducing conditions in gels of NMC-4 immunoprecipitates.

Example 14 - Expression and characterization of von Willebrand factor subunit fragments produced by COS-1 cells transformed with pAD4/G\textsuperscript{459}, pAD4/G\textsuperscript{462} or pAD4/G\textsuperscript{464} plasmids.

Transformation of COS-1 cells by either pAD4/G\textsuperscript{459}, pAD4/G\textsuperscript{462} or pAD4/G\textsuperscript{464} plasmid (collectively the "pAD4/Δ1C plasmids") was accomplished according to the procedure of Example 11. Culture media were analyzed for secreted vWF polypeptide according to the procedure of Example 7, using only NMC-4 for immunoprecipitation.

\textsuperscript{35}S-labelled proteins, prepared according to Example 12, were immunoprecipitated by NMC-4 and run in SDS-polyacrylamide gels under reducing and non-reducing conditions and compared with vWF antigen produced by pAD4/WT and pAD4/Δ3C transformants.

The gels demonstrated that substitution of any one of the 3 cysteines (459, 462, 464) believed responsible for interchain disulfide contacts in native mature subunits prevents the formation of the homodimeric 116 kDa polypeptide characteristic of pAD4/WT transformed
COS-1 cells. These three vWF antigens with a single glycine substitution appear predominantly as monomeric polypeptides of an apparent molecular weight of 52,000 under reducing or non-reducing conditions. That the predominant material has an apparent molecular weight of 52 kDa is strongly suggestive of correct glycosylation by the COS-1 cell transformants duplicating glycosylation seen in the human 52/48 kDa tryptic vWF fragment. Some inadequately glycosylated and/or proteolyzed vWF antigen (molecular weight less than 52 kDa) is also apparent in the gels. The relatively small fraction of pAD4/Δ3C vWF polypeptide which is successfully folded and secreted, thereby presenting an NMC-4 epitope, was shown by the low intensity of the pAD4/Δ3C transformant autoradiograph band of apparent 52,000 molecular weight.

Example 15 - Enhancement of the Affinity of the Recombinant 116 kDa vWF Fragment for Platelet GPIb(α) Receptor Sites in the Presence of Ristocetin

This example demonstrates that the affinity of the recombinant 116 kDa vWF fragment for platelets can be enhanced by reducing the amount of N-linked glycosylation present in the 116 kDa polypeptide.

Stable CHO-K1 transformants containing DNA from pAD5/WT plasmids were incubated overnight (following generally the cell culture procedures of Example 1 and with an initial cell density of about 5 x 10^5 cells/60 mm tissue culture dish) in DMEM containing 10% FCS with 0.5 mM of each nonessential amino acid, 2 mM L-glutamine, and also tunicamycin (from Streptomyces,
product T7765 containing A, B, C and D isomers thereof, Sigma Chemical Co., St. Louis, MO) at 0.8 μg/ml.

The cells were then washed twice with PBS and incubated in DMEM with 0.5 mM of each nonessential amino acid, 2 mM L-glutamine and 0.4 μg/ml tunicamycin for 24 additional hours. The culture medium was harvested and concentrated 300 fold in a centrifugation-filtration apparatus, model Centricon 30, Amicon Co., Lexington, MA.

As a control, medium from stable transformants incubated without tunicamycin (under otherwise identical conditions) was also harvested. The respective abilities of the vWF-derived recombinant 116 kDa dimeric polypeptides (from treated and untreated cultures) to support ristocetin-induced platelet aggregation were compared.

The amount of vWF derived antigen varied from preparation to preparation depending on the precise extent of growth in each tissue culture dish. Based on the ratio of NMC-4 reactivity of particular samples of FCS-free medium derived from treated and untreated cells, respective μl amounts of 300 fold concentrated medium were chosen to reflect equal amounts of vWF antigen for comparison in the ristocetin assay. Equal NMC-4 affinity constants were presumed.

In order to determine the normalizing ratio, between 10 and 100 μl quantities of FCS-free culture medium samples (from treated and untreated cultures) were electrophoresed in SDS-polyacrylamide gels as described in Example 1, after which the bands were
transferred to nitrocellulose sheets for immunoblotting according to a standard procedure. Burnette, et al., A. Anal. Biochem., 112, 195-203 (1981). Detection on the nitrocellulose sheets was accomplished using NMC-4 as primary antibody followed by ¹²⁵I-rabbit anti-mouse IgG as secondary antibody and visualization by autoradiography (see Example 1). For normalization, total vWF antigen reactive with NMC-4 from each culture was determined from densitometric scans of the autoradiographs.

Ristocetin-induced platelet aggregation assays were performed according to the procedure of Example 2 and demonstrated that tunicamycin treated cells produced a NMC-4 reactive antigen having a greater platelet aggregation inducing capability than that produced by untreated cells which generated polypeptides with normal N-linked glycosylation. The comparative aggregation profiles used ristocetin concentrations of 0.5, 0.75 and 1.0 mg/ml.

In addition, the NMC-4 reactive 116 kDa polypeptide material from untreated cells was resolved by Western blotting into multiple species with slightly different electrophoretic mobilities. After treatment with tunicamycin, only a single species was observed. It is thus demonstrated that N-linked glycosylation of the recombinant 116 kDa fragment is heterogeneous and that the level of such glycosylation affects the biological activity of the fragment.
Example 16 - Construction of Mammalian Transformants for the Expression of Monomeric or Dimeric Forms of the Residue 441-730 von Willebrand Factor Subunit Fragment with Reduced Levels of Glycosylation

This example demonstrates the preparation of vWF-derived polypeptides patterned upon the mature subunit 449-728 sequence (the 52/48 kDa fragment), or dimers thereof, but containing less glycosylation than that present in the 52/48 fragment, or dimers thereof, as isolated from circulating plasma vWF.

Mutagenesis of vWF cDNA

One or more particular codons of a cDNA encoding the mature subunit residue 441-730 fragment which encode serine, threonine or asparagine residues thereof may be replaced with codons for other amino acids, such as, for example, alanine or glycine, following the procedures of Examples 1 and 3.

Briefly, the M13mp18 recombinant DNA sequence encoding vWF subunit residues 441-730 and designated pAD3-1 (Example 7) can be cloned into pcDNA1 vector (according to the procedure of Example 9) to generate the pAD4/WT plasmid.

pAD4/WT plasmid can be restricted with EcoRI and SmaI enzymes. As explained in Example 9, pcDNA1 contains an EcoRI site within its polylinker region but no SmaI site. A unique SmaI site (CCCGGG) is contained within the vWF cDNA insert, spanning mature subunit residues 716 (glycine) to residue 718 (glycine).
This approximate 950 base pair EcoRI-SmaI fragment of pAD4/WT can be subcloned into the EcoRI-SmaI site within the polylinker region of M13mp18 phage. The vWF sequence can then be mutagenized to delete or replace one or more serine, threonine, or asparagine codons (encoding potential sites of glycosylation) prior to being reintegrated into the previously restricted pAD4/WT construct, leading to reassembly of the intact residue 441-730 vWF sequence.

A preferred form of mutagenesis follows the procedure of Kunkel, T.A., supra (Example 7) and utilizes a hybridizing oligonucleotide suitable for deleting one or more serine, threonine, or asparagine codons, or alternatively suitable for substituting one or more codons for other amino acids, such as for glycine or alanine. The pcDNA1-derived plasmid containing vWF cDNA which encodes a polypeptide with reduced potential for glycosylation can be designated pAD4/-G.

Following the procedures of Examples 9 and 10, COS-1 cells can be transformed with pAD4/-G plasmids. The polypeptides expressed in this way will form 116 kDa homodimers which compared to the pAD4/WT polypeptides have fewer potential sites for glycosylation. Many other expression plasmid/host cell systems can be used to express the mutant vWF cDNA including notably the pCDM8neo/CHO-K1 system of Example 7.

Expression of Monomeric Fragments
Following the procedure of Example 9, deletion of or substitution for one or more codons encoding one or more of the above mentioned potential glycosylation sites within the 441-730 sequence can be performed with an oligonucleotide which also encodes cys → gly codon changes at, for example, cysteine residue positions 459, 462, and 464. Alternatively, a second round of mutagenesis could be performed in M13mp18 phage to effect the cysteine to glycine mutations.

When reassembled, the pcDNA1 plasmid construct containing cys → gly mutations at vWF subunit positions 459, 462, and 464, and one or more further codon mutations to restrict glycosylation of the encoded vWF polypeptide, can be designated pAD4/Δ3C,-G. This polypeptide, lacking the cysteine residues which stabilize the 116 kDa homodimer (see Examples 12 and 14) will be expressed and secreted from host cells as a monomeric fragment.

Example 17 - Expression and Secretion by Eucaryotic Cells of Other Therapeutic Polypeptides

Example 7 and Examples 12-14 demonstrate that the polypeptide consisting of the 22 residue human vWF signal peptide and the first three amino acids of the human vWF propeptide directs the successful secretion from CHO-K1 and COS-1 cells of the mature vWF subunit fragment, consisting of residues 441-730, which fragment could otherwise only be recovered from host cells by cell lysis.
The amino acid sequence (see SEQ ID NO: 15)

\[ \text{NH}_2- \text{Met-Ile-Pro-Ala-Arg-Phe-Ala-Gly-Val-Leu-Leu-Ala-Leu-Leu-Leu-Ile-Leu-Pro-Gly-Thr-Leu-Cys-Ala-Glu-Gly-Thr-Arg-Gly-Arg-Ser-Thr-CO}_2\text{H} \]

Known signal peptidase cleavage site

and fragments and combinations of fragments thereof will prove useful in the process of directing the secretion into the lumen of the endoplasmic reticulum and, therefore, into the culture medium of eucaryotic host cells of therapeutic polypeptides comprising other regions of the vWF molecule or consisting of other protein species or fragments thereof.

It is believed also that the amino acid sequence comprising \( \text{NH}_2-\text{ala-glu-gly-CO}_2\text{H} \) will facilitate the identification by signal peptidases of a proper cleavage site, when said amino acid sequence is positioned on the C-terminal side of the human vWF signal peptide.

Following the procedures of Example 7, a DNA sequence useful in the expression of a therapeutic polypeptide can be constructed in which the following structural elements would be assembled in a 5' to 3' direction (referring to the coding or nontranscribed strand):

- (A) a sequence of nucleotides suitable for restriction;
- (B) a eucaryotic consensus translation initiation sequence;
- (C) a methionine codon followed by 21 other codons which together would encode the human vWF signal peptide;
(D) a coding sequence corresponding to approximately the first three amino acids of the amino terminal region of the human vWF propeptide;

(E) the coding sequence for the therapeutic polypeptide;

(F) a translation termination codon; and

(G) a sequence of nucleotides suitable for restriction.

This construct may then be inserted into a plasmid or viral expression vector which cloning vehicle may in turn be used to transform suitable eucaryotic host cells from which the therapeutic polypeptide would be expressed.

Example 18 - Preparation of Subsets of the 52/48 kDa Polypeptide

This example is illustrative of the preparation of polypeptides representing embodiments of the invention which are cysteine-deficient subsets derived from the residue 441-733 fragment of vWF subunit. The example is also illustrative of conditions under which such subsets may be expressed from recombinant bacterial host cells. The subsets may be expressed also from recombinant eucaryotic cells, for example, by following the general procedures of Examples 7 and 9. The subsets are capable of interfering with the interaction of multimeric vWF and platelet GPIIb\(\alpha\), that is, they have utility as antithrombotics.

There follows hereafter a description of the preparation of three groups of polypeptides comprising the aforementioned type subsets, with the first group
of subsets being cysteine-free and those of the second and third groups of subsets having but two cysteine residues (five of the cysteine residues having been removed). The subsets of the second and third groups differ in that there is retained either the N-terminal region (second group) or the C-terminal region (third group) of the polypeptide.

Polypeptide Subsets (cysteine-free) of the Residue 441-733 Domain of VWF Subunit

Mutant (fusion) polypeptides consisting of the residue 441-733 sequence, but lacking either the internal G10 (residues 474-488) or D5 (residues 694-708) region, were created using loopout mutagenesis in M13mp18 phage of restriction fragments of p7E constructs and then tested for antithrombotic activity.

Specifically, p7E plasmids were recovered from cultures of E.coli BL21(DE3) using an alkaline cell lysis procedure, Birnboim, H.C. and Doly, J., Nucleic Acids Research, 7, 1513 (1979) followed by purification by CsCl/ethidium bromide equilibrium centrifugation. An XbaI restriction site exists in p7E plasmid (contributed by the parent pET-3A vector) upstream from the T7 transcription promoter. Accordingly, the VWF insert (for residues 441-733) was removed as an XbaI-HindIII restriction fragment for loopout mutagenesis (see Example 1) in M13mp18 phage. Loopout of the G10 region or D5 region, respectively, was accomplished using the following oligonucleotides which represent non-coding strand (transcribed strand) DNA. Shown below the two 3'→5' oligonucleotides are the corresponding coding strands and resultant amino acid sequences.
Oligonucleotide (14) - see SEQ ID NO: 16

3' - GAG TGG CCA CTT CGG CAC TCG GGG TGG TGA - 5'
5' - ctc acc ggt gaa gcc gtt agc ccc acc act - 3'
Leu Thr Gly Glu Ala Val Ser Pro Thr Thr
469 470 471 472 473 489 490 491 492 493
↑
deletion of G10 binding peptide

Oligonucleotide (15) - see SEQ ID NO: 17

3' - CTC TAG CAA TCG ATG CTG TAC CGT GTT CAG - 5'
5' - gag atc gtt agc tac gac atg gca caa gtc -3'
Glu Ile Val Ser Tyr Asp Met Ala Gln Val
689 690 691 692 693 709 710 711 712 713
↑
deletion of D5 binding peptide

DNA sequence analysis was used to confirm that the intended vWF coding sequences were produced. The two mutagenized XbaI-HindIII restriction fragments were then inserted into separate pET-3A plasmids that had been cut with XbaI and HindIII restriction endonuclease and which were thereafter designated p7E/ΔG10 and p7E/ΔD5.

The resultant mutant (fusion) vWF polypeptides were then tested for their ability to bind to GPIbα. Using the assay procedure of Example 6 (inhibition of the binding of LJ-Ib1 antibody to GPIbα in the absence of botrocetin modulator), it was determined that the residue 441-733 fragment, which was expressed from p7E and from which the "G10" peptide sequence was deleted, binds GPIbα. The p7E-derived fusion fragment lacking
the "D5" peptide sequence did not. However, when the experiments were repeated using botrocetin as a modulator of binding (see the method of Example 6), both of the fused subfragments were effective in inhibiting binding by LJ-Ib1, and hence have antithrombotic utility.

Other in vitro assays which can be used to identify vWF-derived polypeptides having antithrombotic activity include inhibition of botrocetin-induced binding of vWF to platelets by the mutant polypeptide (see Example 3), and the inhibition of human platelet agglutination in a system using bovine vWF, but without a modulator such as botrocetin or ristocetin.

Cysteine-deficient Polypeptide Subsets Having N-terminal Deletions

Therapeutic polypeptide subsets effective as antithrombotics have also been prepared which are patterned upon the residue 441-733 vWF subunit fragment, but which contain N-terminal deletions.

Preparation of such polypeptides was accomplished using loopout mutagenesis in M13mp18 phage of the XbaI-HindIII restriction fragment from p5E expression plasmid. Thus, the vWF encoding sequence (p5E) encoded cysteine for residue positions 509 and 695 and glycine at residue positions 459, 462, 464, 471 and 474. p7E sequence is also useful for expression of such antithrombotic polypeptides. Antithrombotic polypeptides equivalent to those expressed from p7E constructs can be made by reduction and alkylation of cysteine residues otherwise contained therein.
The design of oligonucleotides used to create N-terminal deletions in the vWF subunit fragment made reference to DNA sequence of the pET-3A vector that is upstream (5') from the codon encoding vWF residue 441. Expression of the residue 441-733 fragment as an EcoRI-HindIII insert (with both 5' and 3' ends thereof modified by BamHI linkers, Example 1) in pET-3A involves expression also of a twenty residue amino acid sequence (SEQ ID NO:18) that remains attached to the amino terminal of the vWF fragment. This sequence, as shown below, is encoded by vector DNA downstream from the T7 promoter site but does not affect adversely the therapeutic activity of the vWF polypeptide.

initiation codon
↓

Met Ala Ser Met Thr Gly Gly Gln Gln Met
Gly Arg Gly Ser Pro Gly Leu Gln Glu Phe Arg
↓
from EcoRI

It is noted that the EcoRI encoding sequence (Glu-Phe) survived modification with a BamHI linker in the T₄-DNA ligase procedure (Example 1) in this particular case. The corresponding pET-3A vector coding sequence located upstream from the initiating methionine and residue 441 (arginine) is as follows.

Oligonucleotide (16) - see SEQ ID NO: 19
5' - GAA GGA GAT ATA CAT ATG GCT AGC . . .
    Met Ala Ser

Accordingly, generation of N-terminal deletions was accomplished using loopout mutagenesis with a hybridizing oligonucleotide which encodes sequence from the vector (ending at the initiating methionine)
and then the intended N-terminal region of the new vWF polypeptide.

Representative of the oligonucleotides necessary for the preparation of the therapeutic polypeptides is oligonucleotide 17 (SEQ ID NO: 20) which corresponds to non-coding strand (transcribed strand) DNA. Shown below this oligonucleotide are the corresponding coding strand and resultant amino acids.

3' - CCT CTA TAT GTA TAC GTC CTC GCC CCT CCG - 5'  
gga gat ata cat atg cag gag ccg gga ggc  
Met Gln Glu Pro Gly Gly  
474 475 476 477 478 479

Representative of cysteine-deficient polypeptides reflecting such N-terminal deletions are Met·Gln⁷³⁵ to Val⁷³⁸, Met·Thr⁶⁹² to Val⁷³⁸, and Met·Tyr⁵⁰⁷ to Val⁷³⁸. Such polypeptides (and other species having terminal deletion of any subsets of the vWF residue 441-508 sequence that contain one or more cysteine residues) have antithrombotic therapeutic activity. These polypeptides can present also the cysteine 509-695 loop when expressed from p5E constructs.

Cysteine-deficient Polypeptide Subsets Having C-Terminal Deletions

The procedure used to express recombinant bacterial polypeptides using pET-3A vectors results in polypeptides that comprise also a series of amino acids on the C-terminal side of Val⁷³³, the additional residues arising from translation of vector sequence (see SEQ ID NO: 21).

Specifically, residue 441-733 fragments expressed from p5E (or p7E) constructs contain also 22 residues
fused to the C-terminal side of residue 733 (valine) resulting from the expression of vector sequence prior to the first vector stop codon.

This pET-3A vector sequence, which reflects also modification (Example 1) of the HindIII site of the EcoRI-HindIII fragment by a BamHI linker, is (SEQ ID NO: 21):

Val Ser Ser Asp Pro Ala Ala Asn Lys Ala 733
Arg Lys Glu Ala Glu Leu Ala Ala Ala Thr
Ala Glu Gln *
stop codon

In order to prepare an appropriate encoding DNA sequence for vWF polypeptides having C-terminal deletions, loopout mutagenesis was performed in p5E using hybridizing oligonucleotides patterned on non-coding strand DNA. To prepare a polypeptide (using the polypeptide ending at residue Asp^{709} as an example), a hybridizing oligonucleotide was created encoding vWF subunit sequence (for example, from residue 706 to 713) that included also between certain codons thereof (for example, codon 709 and codon 710) the stop codon/reading frame shift sequence 3' - ACT - ACT - T - 5'.

Accordingly, vWF-derived polypeptides were generated that have C-terminal deletions and which terminate at residues 709, 704, 700 and 696 respectively.
Deposit of Strains Useful in Practicing the Invention

Deposits of biologically pure cultures of the following strains were made under the Budapest Treaty with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland. The accession numbers indicated were assigned after successful viability testing, and the requisite fees were paid.

Access to said cultures will be available during pendency of the patent application to one determined by the Commissioner of the United States Patent and Trademark Office to be entitled thereto under 37 C.F.R. §1.14 and 35 U.S.C. §122, or if and when such access is required by the Budapest Treaty. All restriction on availability of said cultures to the public will be irrevocably removed upon the granting of a patent based upon the application and said cultures will remain permanently available for a term of at least five years after the most recent request for the furnishing of samples and in any case for a period of at least 30 years after the date of the deposits. Should the cultures become nonviable or be inadvertently destroyed, they will be replaced with viable culture(s) of the same taxonomic description.

<table>
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<th>Strain/Plasmid</th>
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<tr>
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</tr>
<tr>
<td>E.coli XS127 96.4</td>
<td>ATCC 68407</td>
<td>9/19/90</td>
</tr>
</tbody>
</table>
What is claimed is:

1. A polypeptide patterned upon a parent polypeptide and comprising the amino acid sequence of that fragment of mature von Willebrand factor subunit which begins approximately at residue 441 (arginine) and ends at approximately residue 733 (valine), or any subset thereof, in which one or more of the cysteine residues normally present in the parent polypeptide, or subset thereof, have been deleted and/or replaced by one or more other amino acids, said polypeptide having therefore less tendency than the parent polypeptide, or subset thereof, to form intra or interchain disulfide bonds in aqueous media at a physiological pH.

2. A polypeptide according to Claim 1 which contains residues 509 (cysteine) and 695 (cysteine), wherein one or more of cysteine residues 459, 462, 464, 471, and 474 are deleted or replaced by one or more other amino acids.

3. A polypeptide according to Claim 2 in which each of cysteine residues 459, 462, 464, 471, and 474 is deleted or replaced.

4. A polypeptide according to Claim 2 in which cysteine residues 509 and 695 are covalently linked by a disulfide bond.

5. A polypeptide according to Claim 2 in which each of cysteine residues 459, 462, 464, 471, and 474 is replaced by a single residue of one or more of
alanine, threonine, serine, glycine or asparagine.

6. A polypeptide according to Claim 1 in which each cysteine residue is replaced by glycine.

7. A polypeptide according to Claim 2 in which each cysteine residue is replaced by glycine.

8. A polypeptide according to Claim 1 in which each of cysteine residues 459, 462, 464, 471, 474, 509 and 695 is replaced by a single residue of one or more of alanine, threonine, glycine, serine or asparagine.

9. A polypeptide which consists essentially of any subset or combination of subsets of a polypeptide of Claim 1.

10. A polypeptide according to Claim 1 wherein one or more of the cysteine residues normally present in the parent polypeptide, or subset thereof, have been deleted.

11. A polypeptide according to Claim 10 in which one or more amino acid residues adjacent to a deleted or substituted cysteine residue position have also been deleted or substituted.

12. A polypeptide according to Claim 1 wherein at least one additional residue of lysine and/or of arginine extends from the amino and/or from the carboxy terminus of said polypeptide.
13. A polypeptide according to Claim 1 in which one or more of the free thiol groups thereof are chemically inactivated so as to prevent the disulfide bonding thereof.

14. A polypeptide according to Claim 9 comprising one or more fragments of a mature von Willebrand factor subunit, said polypeptide containing a cysteine residue 509 and a cysteine residue 695 linked by a disulfide bond, and further comprising a domain of said von Willebrand factor subunit which binds to platelet membrane glycoprotein Ib.

15. A polypeptide according to Claim 1 which is glycosylated.

16. A polypeptide according to Claim 1 wherein one or more of cysteine residues 459, 462 and 464 are deleted and/or replaced by one or more other amino acids, and wherein said polypeptide has less tendency than said parent polypeptide to form interchain disulfide bonds.

17. A polypeptide according to Claim 16, containing cysteine residue corresponding to positions 509 and 695 of said fragment or subfragment, in which cysteine residues 509 and 695 are linked by an intrachain disulfide bond.

18. A polypeptide according to Claim 16 in which one or more of the amino acid residues adjacent to a deleted or substituted cysteine position have also been deleted or substituted.
19. A polypeptide according to Claim 16 which is glycosylated.

20. A polypeptide according to Claim 16 in which each of cysteine residues 459, 462 and 464 is replaced by one or more residues of an amino acid chosen from among alanine, threonine, serine, glycine or asparagine, the selection for a replacement at one position being independent of the selection of a replacement at another position.

21. A polypeptide according to Claim 16 in which any two of cysteine residues 459, 462 and 464 are replaced, respectively, by one or more residues of amino acids chosen from among alanine, threonine, serine, glycine, or asparagine, the selection for a replacement at one position being independent of the selection of the replacement at the other position.

22. A polypeptide according to Claim 16 in which any one of cysteine residues 459, 462 and 464 is replaced by one or more residues of amino acids chosen from among alanine, threonine, serine, glycine or asparagine.

23. A polypeptide according to Claim 20 in which cysteine residues 459, 462 or 464 are replaced by single residues of glycine.

24. A polypeptide according to Claim 22 in which a single residue of glycine replaces the substituted cysteine residue.
25. A polymeric structure which inhibits the binding of von Willebrand factor to platelet membrane glycoprotein Ib comprising two covalently linked domains, one of the domains comprising:

a polymer chosen from (A) or (B) below (domain 1):

(A) a polymer having a linear sequence of amino acids which includes the sequence from approximately residue 469 (leucine) to approximately residue 520 (aspartic acid) of mature von Willebrand factor subunit, or any subset thereof; or

(B) a polymer having a linear sequence of amino acids which includes the sequence from approximately residue 469 (leucine) to approximately residue 520 (aspartic acid) of mature von Willebrand factor subunit, or any subset thereof, in which one or more of cysteine residues 471, 474, and 509 are deleted or replaced by one or more other amino acids; and the other domain comprising:

a polymer chosen from (C) or (D) below (domain 2):

(C) a polymer having a linear sequence of amino acids which includes the sequence from approximately residue 689 (glutamic acid) to approximately residue 713 (valine) of mature von Willebrand factor subunit, or any subset thereof; or
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(D) a polymer having a linear sequence of amino acids which includes the sequence from approximately residue 689 (glutamic acid) to approximately residue 713 (valine) of mature von Willebrand factor subunit, or any subset thereof, in which cysteine residue 695 is deleted or replaced by another amino acid.

26. A polypeptide structure which inhibits the binding of von Willebrand factor to platelet membrane glycoprotein Ib, comprising two domains linked by a disulfide bond, one of the domains comprising:

a peptide chosen from (A) or (B) below

(domain 1):

(A) a peptide which includes the sequence of amino acids of mature von Willebrand factor subunit from approximately residue 469 (leucine) to approximately residue 520 (aspartic acid) or any subset thereof; or

(B) a peptide which includes the sequence of amino acids of mature von Willebrand factor subunit from approximately residue 469 (leucine) to approximately residue 520 (aspartic acid) or any subset thereof, in which one or both of cysteine residues 471 and 474 are deleted or replaced by one or more
other amino acids; and the other
domain (domain 2) comprising:
a peptide which includes the sequence of
amino acids of mature von Willebrand
factor subunit from approximately
residue 689 (glutamic acid) to
approximately residue 713 (valine) or
any subset thereof which contains
residue 695; and

wherein said disulfide bond connects cysteine
residue 509 of domain 1 and cysteine residue
695 of domain 2.

27. A DNA sequence encoding the fragment of mature von
Willebrand factor subunit having an amino terminus
at approximately residue 441 (arginine) and a
carboxy terminus at approximately residue 733
(valine) or encoding a subfragment thereof, in
which one or more of the cysteine codons normally
found in said DNA sequence are deleted or replaced
by missense codons.

28. A DNA sequence according to Claim 27 in which the
codons encoding amino acid residues 459, 462, 464,
471, 474, 509, and 695 of the mature von
Willebrand factor subunit are deleted or replaced
by missense codons.

29. A DNA sequence according to Claim 27 in which the
wild-type codons encoding cysteine residues 459,
462, 464, 471 and 474 of the mature von Willebrand
factor subunit are deleted or replaced by missense
codons.
30. A DNA sequence according to Claim 27 in which one or more of the wild-type cysteine codons encoding residues 459, 462 and 464 are deleted or replaced by missense codons.

31. A DNA sequence according to Claim 27 in which each of the missense codons codes for glycine.

32. A DNA sequence according to Claim 27 in which the missense codons code for one or more of alanine, threonine, serine, glycine, or asparagine.

33. A DNA sequence according to Claim 27 which comprises also a restriction endonuclease site at each end of the sequence.

34. A DNA sequence according to Claim 33 in which the restriction endonuclease site preceding the codon of residue 441 is EcoRI and the restriction endonuclease site following the codon of residue 733 is HindIII.

35. A DNA sequence encoding any subset or combination of subsets of the fragment of mature von Willebrand factor subunit having an amino terminus at approximately residue 441 (arginine) and a carboxy terminus at approximately residue 733 (valine), in which one or more of the up to 7 cysteine codons normally found therein are deleted or replaced by missense codons.

36. A DNA sequence according to Claim 30 in which the codons encoding cysteine residues 459, 462 and 464 are each replaced by missense codons.
37. A DNA sequence according to Claim 30 in which a codon encoding one of cysteine residues 459, 462 and 464 is replaced by a missense codon.

38. A DNA sequence according to Claim 30 in which the codons encoding any two of cysteine residues 459, 462 and 464 are replaced by missense codons.

39. A DNA sequence according to Claim 30 in which each cysteine codon for which a substitution is made is replaced by one or more codons chosen from among those which encode alanine, threonine, serine, glycine, or asparagine, the selection for a replacement at any one position being independent of the selection of the replacement at any other position for which a substitution is also made.

40. A DNA sequence according to Claim 39 in which a glycine codon is substituted for any cysteine codon replaced therein.

41. A DNA sequence comprising domains (A), (B) and (C) as follows:

   domain (A) a DNA sequence encoding the von Willebrand factor signal peptide; and downstream therefrom,

   domain (B) a DNA sequence consisting essentially of nine nucleotides and encoding the first three amino acids of the amino terminus region of the von Willebrand factor
propeptide; and downstream therefrom, domain (C) a DNA sequence encoding all or part of the 2,050 amino acid sequence of the mature von Willebrand factor subunit.

42. A DNA sequence according to Claim 41 in which the DNA subsequence, domain (C) thereof, corresponding to mature von Willebrand factor subunit DNA encodes an amino acid sequence from approximately residue 441 (arginine) to approximately residue 730 (asparagine).

43. A DNA sequence according to Claim 41 in which the DNA subsequence, domain (C) thereof, corresponding to mature von Willebrand factor subunit DNA encodes discontinuous subsequences of mature von Willebrand factor subunit amino acid primary structure.

44. A polypeptide which is capable of directing the transport of additional polypeptide sequence across the membrane of the endoplasmic reticulum of a cell and which is comprised of a domain (A) and a domain (B) as follows:

\[
\text{domain (A)} \quad \text{any subset of the signal peptide of human von Willebrand factor subunit which signal peptide is capable of being recognized by the endoplasmic reticulum and/or by translocation receptors which complex with}
\]

\[
\text{domain (B)}
\]
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the endoplasmic reticulum
and/or the signal peptide; and

domain (B)
a peptide sequence consisting
essentially of up to the first
ten residues of the amino
terminal end of von Willebrand
factor propeptide;

said domain (B) being connected by amide linkage to the
carboxy terminus of domain (A) and capable of being
connected by amide linkage to the amino terminus of
said additional polypeptide sequence; which polypeptide
comprising domain (A) and domain (B) contains a
sufficient subset of the sequence of the human von
Willebrand factor signal peptide and propeptide to
permit intracellular cleavage of said polypeptide
containing additional polypeptide sequence in a manner
such that the therapeutic activity of the additional
polypeptide sequence is retained in whole or part.

45. A cloning vehicle which contains a DNA sequence
according to Claim 27.

46. A cloning vehicle comprising essentially M13mp18
bacteriophage which contains a DNA sequence
according to Claim 27.

47. A cloning vehicle which contains a DNA sequence
according to Claim 30.

48. A biologically functional expression plasmid or
viral expression vector, containing DNA encoding
for a fragment of mature von Willebrand factor
subunit having an amino terminus at approximately
residue 441 (arginine) and a carboxy terminus at
approximately residue 733 (valine), or a subfragment thereof, in which one or more of the cysteine codons normally present in the encoding DNA are deleted or replaced by missense codons, which plasmid or vector is capable of being replicated in a host cell and directing expression therein of said vWF subunit fragment or subfragment.

49. An expression plasmid or viral expression vector according to Claim 48 in which the codons thereof encoding cysteine residues 459, 462, 464, 471 and 474 of the mature von Willebrand factor subunit within said plasmid or vector are deleted or replaced by missense codons.

50. An expression plasmid or viral expression vector according to Claim 48 in which the codons thereof encoding cysteine residues 459, 462, 464, 471, 474, 509 and 695 of the mature von Willebrand factor subunit within said plasmid or vector are deleted or replaced by missense codons.

51. An expression plasmid according to Claim 48 which is selected from a group consisting of pBR 322, pET-1 through pET-7, and any constructs derived therefrom.

52. An expression plasmid or viral expression vector capable of directing, in eucaryotic cells, the expression therein and secretion therefrom of a fragment of mature von Willebrand factor subunit having an amino terminus at approximately residue 441 (arginine) and a carboxy terminus at
approximately residue 730 (asparagine), or a subfragment thereof, said plasmid or vector therefore containing a transcriptional promoter, followed downstream by a DNA sequence encoding said fragment or subfragment, and a signal sequence positioned upstream from and in proper reading frame with said encoding DNA sequence, said signal sequence directing and/or facilitating the secretion of the fragment or subfragment from the eucaryotic cell.

53. An expression plasmid or viral expression vector according to Claim 48 capable of directing, in eucaryotic cells, the expression therein and secretion therefrom of a fragment of the mature von Willebrand factor subunit having an amino terminus at approximately residue 441 (arginine) and a carboxy terminus at approximately residue 730 (asparagine), or a subfragment thereof, in which one or more of cysteine residues 459, 462 and 464 are replaced by single residues of glycine, said plasmid or vector therefore containing a transcriptional promoter, followed downstream by a DNA sequence encoding said fragment or subfragment, and a signal sequence positioned upstream from and in proper reading frame with said encoding DNA sequence, said signal sequence directing and/or facilitating the secretion of the fragment or subfragment from the eucaryotic cell.

54. A recombinant host transformed with an expression plasmid or viral expression vector of Claim 48.
55. A recombinant host according to Claim 54 wherein said host is a prokaryot selected from *Escherichia*, or *Bacillus*, or a eucaryot selected from a group consisting of yeast (*Saccomyces*), cultured insect cells, and cultured mammalian cells.

56. A recombinant host according to Claim 54 wherein the host is *E. coli*, strain BL21(DE3), and the expression plasmid therein is pET-3A.

57. A viral expression vector according to Claim 48 which is selected from the group consisting of the baculovirus *Autographa californica* nuclear polyhedrosis virus, and retroviruses.

58. A recombinant eucaryotic host cell transformed with an expression plasmid or viral expression vector according to Claim 52.

59. A process for producing from DNA which encodes that fragment of mature von Willebrand factor subunit comprising essentially the amino acid sequence from approximately residue 441 (arginine) to approximately residue 733 (valine), or which encodes any subfragment thereof, a mutant von Willebrand factor fragment, or subfragment thereof, which contains fewer cysteine residues than that of the comparable non-mutant amino acid sequence, and which process comprises culturing a host organism transformed with a biologically functional expression plasmid which contains a mutant DNA sequence encoding a portion of said von Willebrand factor subunit under conditions which
effect expression of the mutant von Willebrand factor fragment, or subfragment, by the host organism and recovering said fragment or subfragment therefrom.

60. A polypeptide produced by the process of Claim 59.

61. A mutant polypeptide patterned upon a parent polypeptide containing a predetermined number of cysteine residues which parent polypeptide further comprises the amino acid sequence of that fragment of mature von Willebrand factor subunit which begins approximately at residue 441 (arginine) and ends at approximately residue 733 (valine), or any subset thereof, which mutant polypeptide contains fewer cysteine residues than said predetermined number and which is produced by mutagenesis of a nucleotide sequence coding for the parent polypeptide.

62. A mutant polypeptide according to Claim 61, in which one or more amino acid residues adjacent to a deleted or substituted cysteine position have also been deleted and/or substituted.

63. A process for producing a mutant polypeptide patterned upon a parent polypeptide which parent comprises the sequence of amino acids from approximately residue 441 (arginine) to approximately residue 733 (valine) of mature von Willebrand factor subunit or a subset of said sequence, which mutant polypeptide differs from the parent polypeptide in that one or more of the cysteine residues of said parent are deleted or
replaced by one or more amino acid residues chosen from alanine, threonine, serine, glycine or asparagine and which process comprises providing a nucleotide sequence which codes for the parent DNA sequence, preparing a mutant nucleotide sequence derived therefrom in which one or more of the codons corresponding to the cysteine residues of the parent polypeptide are deleted or mutated, and then culturing a host organism transformed with a biologically functional expression plasmid or viral expression vector which contains said mutant DNA sequence under conditions which effect expression of the mutant polypeptide by the host organism, and recovering said polypeptide therefrom.

64. A process for producing from DNA corresponding to that fragment of mature von Willebrand factor subunit comprising essentially the amino acid sequence from approximately residue 441 (arginine) to approximately residue 730 (asparagine), or a subfragment thereof containing one or more of residue positions 459, 462, and 464, a biologically active dimer of said subunit fragment or subfragment which process comprises the steps of:

(A) providing a DNA sequence encoding the subunit fragment or subfragment which contains upstream from the fragment encoding region thereof and in proper reading frame therefor, a signal peptide sequence; and

(B) inserting the DNA sequence into a suitable vector to create a construct comprising an expression plasmid or viral expression
vector, which construct is capable of
directing the expression in, and secretion
from, eucaryotic cells of said subunit
fragment or subfragment; and

(C) transforming a eucaryotic host cell with said
expression plasmid or viral expression
vector; and

(D) culturing said transformed host cell under
conditions that cause expression within the
host cell and secretion therefrom of the
dimeric form of the subunit fragment or
subfragment, and under which the monomeric
subunit fragment or subfragment assumes a
tertiary structure suitable for dimerization
and the dimerization thereof, and under which
there is effected glycosylation of said
monomeric subunit fragment or subfragment or
of a dimeric form thereof.

65. A dimeric polypeptide prepared by the process of
Claim 64 which is glycosylated and has an apparent
molecular weight as measured by SDS-polyacrylamide
gel electrophoresis of about 116 kDa.

66. A process for producing from DNA corresponding to
that fragment of mature von Willebrand factor
subunit comprising essentially the amino acid
sequence from approximately residue 441 (arginine)
to approximately residue 730 (asparagine), a
biologically active monomer of said subunit
fragment having an apparent molecular weight by
SDS-polyacrylamide gel electrophoresis of
approximately 52 kDa which process comprises the
steps of:
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(A) providing a DNA sequence encoding the subunit fragment which contains upstream from the fragment encoding region thereof, and in proper reading frame therefor, a signal peptide sequence;

(B) mutagenizing the DNA sequence to reduce the number of cysteine codons capable of specifying cysteine residues normally involved in interchain disulfide contacts;

(C) inserting the DNA sequence into a suitable vector to create a construct comprising an expression plasmid or viral expression vector, said construct being capable of directing the expression in and secretion from eucaryotic cells of said monomeric subunit fragment;

(D) transforming a eucaryotic host cell with said construct; and

(E) culturing said transformed host cell under conditions which cause expression within and secretion from said host cell of the monomeric subunit fragment, said conditions also permitting glycosylation of said fragment.

67. A process for producing from DNA corresponding to a fragment or combination of fragments of von Willebrand factor a biologically active polypeptide structure which process comprises the steps of:

(A) providing a DNA sequence encoding the von Willebrand factor fragment or fragments which further contains upstream from the fragment encoding region thereof, and in proper
reading frame therefor, a DNA subsequence encoding a polypeptide according to Claim 44; and

(B) inserting the DNA sequence into a suitable vector to create a construct comprising an expression plasmid or viral expression vector, which construct is capable of directing the expression in and secretion from eucaryotic cells of said subunit fragment or fragments; and

(C) transforming a eucaryotic host cell with said expression plasmid or viral expression vector; and

(D) culturing said transformed host cell under conditions which cause expression within and secretion from the host cell of said polypeptide structure which conditions further permit the glycosylation thereof.

68. A process for producing from DNA a therapeutic polypeptide which process comprises the steps of:

(A) providing a DNA sequence encoding the therapeutic polypeptide and which further contains upstream from the polypeptide encoding region thereof, and in proper reading frame therefor, a DNA sequence corresponding to a polypeptide according to Claim 44; and

(B) inserting the resultant DNA sequence into a suitable vector to create a construct comprising an expression plasmid or viral expression vector, which construct is capable of directing the expression in and secretion
from eucaryotic cells of said therapeutic polypeptide; and

(C) transforming a eucaryotic host cell with said expression plasmid or viral expression vector; and

(D) culturing said transformed host cell under conditions which cause expression within and secretion from said host cell of the therapeutic polypeptide.

69. An antibody which is specific for von Willebrand factor subunit, or any polypeptide comprising one or more subsets thereof, which antibody is made by a process of immunizing animals with a polypeptide according to Claim 1 and then isolating the specified antibodies generated thereby.

70. An antibody which is specific for von Willebrand factor subunit, or any polypeptide containing a subset thereof, in which the epitope for said antibody is dependent upon the existence of a disulfide bond between two cysteine residues in said subunit, or in a polypeptide containing a subset of said subunit, which cysteine residues are or are equivalent to subunit residues 509 and 695, and which antibody is made by a process of immunizing animals with a polypeptide according to Claim 4 and then isolating the specified antibodies generated thereby.

71. A therapeutic composition comprising one or more polypeptides according to Claim 1 effective to inhibit binding of von Willebrand Factor to
platelets, and a pharmaceutically acceptable carrier.

72. A method of inhibiting platelet activation and/or aggregation which comprises contacting platelets with an effective amount of a composition according to Claim 71.

73. A method of inhibiting adhesion of platelets to surfaces which comprises contacting platelets with an effective amount of a composition according to Claim 71.

74. A method of inhibiting thrombosis in a patient which comprises administering to such patient an effective amount of a composition according to Claim 71.

75. A method of treating von Willebrand disease in a patient comprising administering to such patient an effective amount of a composition according to Claim 65.

76. A polypeptide according to Claim 1 capable of binding to collagen, heparin-like glycosaminoglycans or proteoglycans.

77. A mutant polypeptide formed by mutagenesis of a nucleotide sequence encoding all or part of a parent polypeptide, the parent polypeptide comprising a sequence of amino acids having substantial sequence homology with the "A₁" domain of von Willebrand factor and further containing a predetermined number of cysteine residues, wherein
said mutant polypeptide contains cysteine residues forming an intrachain disulfide bond of the parent polypeptide but fewer cysteines than said predetermined number.

78. A mutant polypeptide formed by mutagenesis of a nucleotide sequence encoding all or part of a parent polypeptide, the parent polypeptide comprising a sequence of amino acids having substantial sequence homology with the "A_1" domain of von Willebrand factor, wherein said mutant polypeptide contains one or more additional cysteine residues not found in the parent polypeptide.

79. A process for producing from DNA corresponding to that monomeric fragment of mature von Willebrand factor subunit comprising essentially the amino acid sequence from approximately residue 441 (arginine) to approximately residue 730 (asparagine), or a subfragment thereof, containing one or more of residue positions 459, 462, and 464, a biologically active dimer of said monomeric fragment or subfragment which process comprises the steps of:

(A) constructing a DNA sequence encoding the monomeric fragment or subfragment which further contains upstream from the fragment encoding region thereof and in proper reading frame therefor, a signal peptide sequence;

(B) inserting the DNA sequence into a suitable vector to create a construct comprising an expression plasmid or viral expression vector, said construct being capable of
directing the expression in, and secretion from, eucaryotic cells of said monomeric fragment or subfragment;

(C) transforming a eucaryotic host cell with said construct;

(D) culturing said transformed host cell under conditions that cause expression within the host cell and secretion therefrom of the dimeric form of the monomeric fragment or subfragment and under which the monomeric fragment or subfragment assumes a tertiary structure suitable for the dimerization thereof, and under which there is effected glycosylation of said monomeric subunit fragment or subfragment or of a dimeric form thereof.

80. A mutant polypeptide patterned upon a parent polypeptide which comprises the amino acid sequence of that fragment of mature von Willebrand factor subunit which begins approximately at residue 449 (valine) and ends at approximately residue 728 (lysine), or a dimer thereof, from which parent one or more serine, threonine or asparagine residues which are sites of O- or N-linked glycosylation have been deleted or replaced by one or more other amino acids, said mutant polypeptide having less glycosylation when said mutant polypeptide is expressed from recombinant DNA in a host eucaryotic cell than the species of the parent polypeptide having an apparent molecular weight of 52 kDa, as measured by SDS-polyacrylamide gel electrophoresis.
81. A DNA sequence which encodes a mutant polypeptide according to Claim 80.

82. A method of preventing or treating thrombosis in a patient which comprises administering to such patient an effective amount of a therapeutic composition comprising

(A) a pharmaceutically acceptable carrier; and

(B) a polypeptide patterned upon the amino acid sequence from approximately residue 449 (valine) to approximately residue 728 (lysine) of mature von Willebrand factor subunit, which polypeptide contains less glycosylation than that found attached to the 449-728 fragment as isolated from circulating mature vWF, having an apparent molecular weight by SDS-polyacrylamide electrophoresis of 52 kDa.

83. A method of treating hemorrhage in a von Willebrand disease patient which comprises administering to such patient an effective amount of a therapeutic composition comprising

(A) a pharmaceutically acceptable carrier; and

(B) a dimeric 116 kDa polypeptide patterned upon the amino acid sequence from approximately residue 449 (valine) to approximately residue 728 (lysine) of mature von Willebrand factor subunit, which dimeric polypeptide contains less glycosylation than that found in the
comparable disulfide-bonded sequence region of fully glycosylated circulating mature vWF and/or contains less than the maximum glycosylation of said 116 kDa disulfide-bonded region as determined by Titani, K. et al., Biochemistry, 25, 3171 (1986).

84. A process for treating a eucaryotic host cell, which contains a DNA sequence encoding with respect to mature von Willebrand factor subunit only that fragment thereof comprising approximately residues 449-728, to limit the glycosylation of the vWF fragments, including dimers thereof, expressed therein comprising adding to the culture medium of said host cells tunicamycin in an amount sufficient to limit said glycosylation.

85. A process for treating a polypeptide according to Claim 1 with an enzyme capable of removing from said polypeptide one or more carbohydrate moieties for the purpose of improving the therapeutic potency of the polypeptide which process comprises adding said enzyme to a sample of said polypeptide under conditions which permit sufficient activity of said enzyme.

86. A process for producing from DNA a therapeutic polypeptide comprising:

(A) providing a DNA sequence encoding the therapeutic polypeptide which contains upstream from the therapeutic polypeptide-encoding region thereof, and in proper
reading frame therefor, a DNA sequence which itself corresponds to a signal peptide and directly downstream therefrom a semipolar or polar spacer sequence;

(B) inserting the resultant DNA sequence into a suitable vector to create a constant comprising an expression plasmid or viral expression vector which is capable of directing the expression in and secretion from eucaryotic host cells of said therapeutic polypeptide;

(C) transforming a eucaryotic host cell with said constant; and

(D) culturing said transformed host cell under conditions which cause expression within and secretion from said host cell of the therapeutic polypeptide.
Glu Asp Cys Pro Val Cys Glu Val Ala Gly Arg Arg Phe Ala Ser Gly Lys Lys Val Thr
440
Leu Asn Pro Ser Asp Pro Glu His Cys Gln Ile Cys His Cys Asp Val Val Asn Leu Thr
460
Cys Glu Ala Cys Gln Glu Pro Gly Gly Leu Val Val Val Pro Pro Thr Asp Ala Pro Val Ser
480
CCC-ACC-ACT-CTG-TAT-GTG-GAG-GAC-ATC-TCG-GAA-CCG-CCG-ATT-GAC-ATC-ATG-GTC-CAG
Pro Thr Thr Leu Tyr Val Glu Asp Ile Ser Glu Pro Pro Leu His Asp Phe Tyr Cys Ser
500
AGG-CTA-CTG-GAC-CTG-GTC-TTC-CTG-GAT-GGC-TCC-TCC-AGG-CTG-TCC-GAG-GCT-GAG-TTT-
Arg Leu Leu Asp Leu Val Phe Leu Leu Asp Gly Ser Ser Arg Leu Leu Ser Glu Ala Glu Phe
520
FIG. 1A
TCC-CGG-AAC-TTT-GTC-CGC-TAC-GTC-CAG-GGC-CTG-AAG-AAG-AAG-GTC-ATT-GTG-ATC-CCG-
Ser Arg Asn Phe Val Arg Tyr Val Gln Gly Leu Lys Lys Lys Lys Val Ile Val Ile Pro 640

GTG-GGC-ATT-GGG-CCC-CAT-GCC-AAC-CTC-AAG-CAG-ATC-CCG-CTC-ATG-GAG-AAG-CAG-GCC-CCT-
Val Gly Ile Gly Pro His Ala Asn Leu Lys Gln Ile Arg Leu Ile Glu Lys Gln Ala Pro 660

GAG-AAC-AAG-GCC-TTC-CTG-AGC-AGT-GTG-GAT-GAG-CTG-GAG-CAG-CAA-AGG-GAC-GAG-ATC-
Glu Asn Lys Ala Phe Val Leu Ser Ser Val Asp Glu Leu Glu Gln Gln Arg Asp Glu Ile 680

GTT-AGC-TAC-CTC-TGT-GAC-CTT-GCC-CCT-GAA-GCC-CCT-CCT-CTC-CTG-CCC-CCC-CAC-ATG-
Val Ser Tyr Leu Cys Asp Leu Ala Pro Glu Ala Pro Pro Pro Thr Leu Pro Pro His Met 700

GCA-CAA-GTC-ACT-CTG-GGC-CGG-GGG-CTC-TG-GTT-TCG-ACC-CTG-GGG-CCC-AAG-AGG-AAC-
Ala Gln Val Thr Val Gly Pro Gly Leu Leu Gly Val Ser Thr Leu Gly Pro Lys Arg Asn 720

TCC-ATG-GTT-CTG-GAT-GTG-GCG-TTC-GTC-CTG-GAA-GGA-TCG-GAC-AAA-ATT-GGT-GAA-GCC-GAC-
Ser Met Val Leu Asp Val Ala Phe Val Leu Glu Gly Ser Asp Lys Ile Gly Glu Ala Asp 740

FIG. 1C
FIG. 2

FIG. 3

SUBSTITUTE SHEET
# INTERNATIONAL SEARCH REPORT

**International Application No:** PCT/US91/07756

## I. CLASSIFICATION OF SUBJECT MATTER

(classification symbols apply, indicate all)

**IPC(5):** 007K 13/00, 3/00, 15/00, 17/00; C120/1/26; C12F 21/06

**U.S.CL.:** 530/324, 350, 356, 403, 807, 808; 435/13, 69.14

## II. FIELDS SEARCHED

**Minimum Documentation Searched**

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**Documentation Searched other than Minimum Documentation**

to the Extent that such Documents are Included in the Fields Searched

## APS TEST SEARCH, BIOSIS, CAS

### III. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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* Special categories of cited documents: *A* document defining the general state of the art which is not considered to be of particular relevance; *E* earlier document but published on or after the international filing date; *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified); *O* document relating to an oral disclosure, use, exhibition or other means; *P* document published prior to the international filing date but later than the priority date claimed. *Y* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention; *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step; *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art; "A" document member of the same patent family.

## IV. CERTIFICATION

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<td>28 JAN 1992</td>
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**International Searching Authority:** ISA/US

**Signature of Authorized Officer:**

[Signature]

Avis Davenport
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<td>J.A. Parsons, &quot;Peptide Hormones&quot;. Published June 1976 by University Park Press (MO) see pages 1-4, especially page 6.</td>
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