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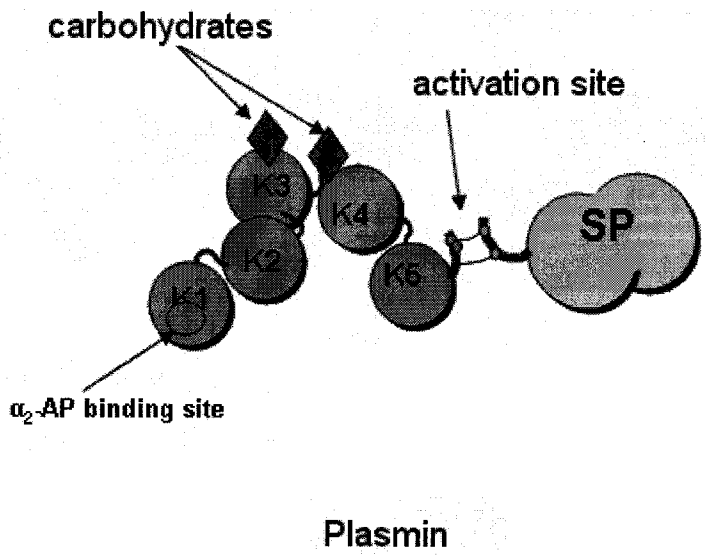
(54) **Title:** RECOMBINANTLY MODIFIED PLASMIN

FIG. 1



(57) **Abstract:** Polynucleotides and polypeptides relating to a recombinantly-modified plasmin(ogen) molecule are provided. The plasmin(ogen) molecule has a single kringle domain N-terminal to the activation site present in the native human plasminogen molecule, combined such that no foreign sequences are present, and exhibits lysine-binding and significant enzymatic characteristics associated with the native enzyme.

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RECOMBINANTLY MODIFIED PLASMIN

Cross-Reference to Related Applications

This application claims priority under 35 USC §119 to U.S. Provisional Application 60/991,148, filed November 29, 2007, incorporated herein by reference.

Background of the Invention

Human plasminogen is a single-chain protein containing 791 amino acid residues. Activation of plasminogen to plasmin results from a single cleavage of the Arg561-Val562 peptide bond in the zymogen. The resulting plasmin molecule is a two-chain, disulfide-linked serine protease with trypsin-like specificity (cleaves after Lys and Arg).

The amino-terminal heavy chain of plasmin (residues 1-561, ~60 kDa) is composed of five kringle domains, each containing approximately 80 amino acid residues. The kringle domains are responsible for the regulatory properties of plasminogen, such as interaction with activation inhibitors, *e.g.*, Cl^{-1} ions; with activation stimulators, *e.g.*, ε-aminocaproic acid; with mammalian and bacterial cells; and with other proteins, such as plasmin physiological substrate fibrin and plasmin inhibitor α2-antiplasmin. Of all five kringles, kringle 1 is one of the most multi-functional: its lysine-binding activity has been shown to be responsible for plasmin interaction with α2-antiplasmin and fibrin. See Wiman, B., *et al.*, *Biochim. Biophys. Acta 579*:142-154 (1979); and Lucas, M.A., *et al.*, *J. Biol. Chem. 258*:4249-4256 (1983).

The C-terminal light chain of plasmin (residues 562-791, ~25kDa) is a typical serine protease, homologous to trypsin and containing the classic serine protease catalytic triad: His603, Asp646 and Ser741. Plasminogen contains 24 disulfide bridges and 2 glycosylation sites on Asn289 and Thr346.

The limited proteolysis of plasminogen by elastase has been shown to result in three major fragments (Sottrup-Jensen, L., *et al.*, *Prog. Chem. Fibrinol. Thrombol.*, *3*:191-209 (1978)). First fragment, K1-3, includes the first three kringles and can be isolated in two versions, Tyr80-Val338 and Tyr80-Val354. The second fragment, K4, corresponds to the fourth kringle and includes residues Val355-Ala440. The last, C-terminal fragment (the so-called mini-plasminogen) includes residues Val443-Asn791

and consists of the fifth kringle and the serine protease domain. Mini-plasminogen can be activated in the same way as plasminogen, forming mini-plasmin.

Because of the complex structure of the full-length plasminogen molecule, bacterial expression systems have not proven useful for recombinant plasminogen production. Plasminogen is produced in the form of insoluble inclusion bodies and is not re-foldable from that state. Further, the expression of plasminogen in mammalian cells is complicated by intracellular activation of plasminogen into plasmin and the resulting cytotoxicity. Production of fully active plasminogen using insect cells is possible, however, this system is not suitable for large-scale production due to low yield. Further, as with any recombinant protein scheme, the potential exists for encountering immunogenicity problems in the subject receiving the therapeutic recombinant protein.

Immunogenicity can be a barrier to the effective and/or efficient utilization of certain recombinant protein therapeutic schemes. Immunogenicity is a complex series of responses to a substance (*e.g.,* the chemical structure of a protein including the amino acid sequence) that is perceived as foreign and may include production of neutralizing and non-neutralizing antibodies, formation of immune complexes, complement activation, mast cell activation, inflammation, and anaphylaxis. Immunogenicity may limit the efficacy and safety of a protein therapeutic in multiple ways. Efficacy can be reduced directly by the formation of neutralizing antibodies. Efficacy may also be reduced indirectly, as binding to either neutralizing or non-neutralizing antibodies typically leads to rapid clearance from serum. Severe side effects and even death may occur when an immune reaction is raised. One special class of side effects results when neutralizing antibodies cross-react with an endogenous protein and block its function.

Accordingly, a modified recombinant protein, possessing the desirable characteristics (*e.g.,* regions with native-like chemical structures) of plasmin/plasminogen while lacking certain negative characteristics and being capable of production in recombinant protein expression systems including bacterial cells in substantial quantities, is desirable.

Summary of the Invention

In one aspect, the present invention provides a polynucleotide comprising a nucleotide sequence encoding a polypeptide having

a) a single N-terminal kringle domain homologous to a kringle domain of native human plasminogen, wherein the last four amino acid residues within the kringle domain are V,P,Q, and C; and

b) a C-terminal domain activation site and serine protease domain homologous to the corresponding domains in human plasminogen; wherein the polypeptide binds to immobilized lysine.

In another aspect, the present invention provides a polypeptide comprising:

a) a single N-terminal kringle domain homologous to a kringle domain of native human plasminogen, wherein the last four amino acid residues within the kringle domain are V,P,Q, and C; and

b) a C-terminal domain activation site and serine protease domain homologous to the corresponding domains in human plasminogen; wherein the polypeptide binds to immobilized lysine.

In other aspects, the present invention provides an expression vector comprising a polynucleotide of the present invention. In one embodiment, the polynucleotide comprises a nucleotide sequence as shown in SEQ ID NO:1.

In some aspects, the present invention provides a cultured cell comprising an expression vector comprising a polynucleotide of the present invention. In one embodiment, the polynucleotide comprises a nucleotide sequence as shown in SEQ ID NO:1. In another embodiment, the cultured cell is a prokaryotic organism. In one embodiment, the prokaryotic organism is *E. coli*.

In one aspect, the present invention provides a method for making one or more recombinant plasmin polypeptides. The method comprises:

a) providing a polypeptide having a single N-terminal kringle domain homologous to a kringle domain of native human plasminogen, wherein the last four amino acid residues within the kringle domain are V,P,Q, and C; and a C-terminal domain activation site and serine protease domain homologous to the corresponding domains in human plasminogen; wherein the polypeptide binds to immobilized lysine; and

b) contacting the polypeptide provided in step a) with a protease under conditions sufficient to cleave one or more peptide bonds thereby forming the one more recombinant plasmin polypeptides. In one embodiment, providing comprises expressing an open reading frame having a sequence corresponding to the sequence as shown in SEQ ID NO:1, or a degenerate variant thereof, in a suitable host. In another embodiment, the polypeptide has an amino acid sequence as shown in SEQ ID NO:2.

Brief Description of the Drawings

Figure 1 is a schematic representation of native plasmin after activation by proteolytic cleavage. K1-K5 are kringle regions 1-5; and SP is the serine protease domain. "α2-AP" is the α_2-antiplasmin binding site on kringle 1.

Figure 2 is a schematic representation of a plasminogen deletion mutant of the invention using the same nomenclature as in Figure 1, and showing the deletion of K2-5.

Figure 3 shows the amino acid sequence of human plasminogen, showing the 19-residue leader sequence numbered as –19 to –1, and the plasminogen sequence shown as residues 1-791 (see SEQ ID NO:3, the cDNA sequence for human plasminogen; and SEQ ID NO:4, the encoded amino acid sequence, as shown in Figure 3). A number of features are shown, including the following: one embodiment of the (TAL6003)-plasminogen sequence (shaded); kringle domains 1-5 (double underscore); glycosylations sites Asn289 and Thr346 (in bold); the Arg-Val activation site ($R^{561}V^{562}$ in bold); and lysine-binding sites in kringle 1 (in underscore and with specific position numbering).

Figure 4 shows polypeptide sequence comparisons (*i.e.*, a gap alignment) between the five kringle domains (1-5) of native human plasmin(ogen). Amino acid residues that are identical to those of the same relative position in kringle 1 are shown in underscore.

Figure 5 shows a 8-25% gradient SDS-PAGE of plasma-derived plasmin (Lane 1= non-reduced (NR); Lane 2 = reduced (R)) and (TAL6003)-plasmin (Lane 3 = non-reduced (NR); Lane 4 = reduced (R)) preparation. Streptokinase activation of plasma-derived plasminogen and (TAL6003)-plasminogen into native plasmin and recombinant (TAL6003)-plasmin, respectively, results in the formation of two bands corresponding to the kringle and the serine protease domains. Accordingly, following

incubation with the reducing agent dithiotreitol (DTT) prior to electrophoresis, plasma-derived plasmin and (TAL6003)-plasmin, which are a single band on a non-reduced gel, reduce to two bands corresponding to kringle 1 (lower band) and the serine protease domain (upper band) in the same non-reduced gel.

Figure 6 is a graphic representation of activation of (TAL6003)-plasminogen by streptokinase.

Figure 7 is a chromatogram showing binding of (TAL6003)-plasminogen to lysine-SEPHAROSE 4B: 0.5 mg of purified (TAL6003)-plasminogen was applied on the lysine-SEPHAROSE 4B column (1x3 cm) equilibrated with Tris-buffered saline, pH 7.4. Bound protein was eluted from the column by a 0-20 mM gradient of ε-aminocaproic acid (ε-ACA) as a single peak. The absorbance at 280 nm and the concentration of ε-ACA, as a function of the effluent volume are presented on the graph.

Figure 8 shows binding of (TAL6003)-plasminogen to fibrin as assessed by its subsequent activation by tPA and resulting clot lysis.

Figure 9 shows *in vitro* comparison of the thrombolytic efficacy of (TAL6003)-plasmin with plasma-derived plasmin.

Figure 10 illustrates disulfide bonding pattern of (TAL6003)-plasmin (SEQ ID NO:2). In the figure, (X) represents the amino acid sequence RDVVLFEK.

Description of the Invention

The present inventors have discovered novel recombinant plasminogen polypeptides, or variants thereof, herein referred to as (TAL6003)-plasminogens that have native plasminogen-like features despite deletion of 4 kringles from its structure. These (TAL6003)-plasminogens, or variants thereof, are zymogens that are capable of becoming activated to functional plasmin enzymes (herein referred to as (TAL6003)-plasmins) following an activation event that at least involves proteolytic cleavage of an Arg-Val peptide bond located between the kringle domain and the serine protease domain of the zymogen.

The (TAL6003)-plasminogen, or a variant thereof, of the present invention has fibrin- and antiplasmin-binding as well as activation properties of full-length native human plasminogen. Further, the (TAL6003)-plasminogen has a number of novel and desirable features including high level expression in recombinant production and

certain protein chemical structures identical or very similar to naturally occurring forms of human plasma-derived plasminogen.

The (TAL6003)-plasmin(ogen)s according to the present invention can be characterized at least by the following:

i) the lower molecular weights (*e.g.*, in one embodiment about 36,911 to about 37,039 Da) of (TAL6003)-plasmins created following activation of (TAL6003)-plasminogens result in increased specific activity (per mg of protein);

ii) the lack of at least two glycosylation sites found in the native protein (see Figure 3, *i.e.*, N^{289} and T^{346}), combined with the relatively low molecular weights, facilitates recombinant production of this protein using relatively inexpensive bacterial and yeast expression systems;

iii) (TAL6003)-plasminogens can be activated by plasminogen activators tPA, urokinase, and streptokinase;

iv) the presence of the single N-terminal kringle domain homologous to a kringle domain of native human plasminogen preserves the fibrin-binding properties of plasmin which are important for thrombolytic efficacy;

v) the presence of α2-antiplasmin-binding sites on the single N-terminal kringle domain homologous to a kringle domain of native human plasminogen allows the (TAL6003)-plasmins to be inhibited rapidly by this physiological inhibitor of plasmin (a feature which can prevent bleeding);

vi) the smaller size of the (TAL6003)-plasmins can facilitate their inhibition by α_2-macroglobulin, further lessening the chance of bleeding complications relative to native plasmin. In particular embodiments, the absence of kringle 5, which retains the primary binding site for intact, undigested fibrin(ogen), can allow use of the (TAL6003)-plasmins with reduced depletion of circulating fibrinogen;

vii) the presence of a single N-terminal kringle domain homologous to a kringle domain of native human plasminogen, wherein the last four amino acid residues within the kringle domain are V,P,Q, and C, provides a native-like linkage to the serine protease domain (*i.e.*, a linkage similar to the naturally occurring domain juncture between the kringle 5 domain and the serine protease domain of human plasminogen); and

viii) following expression of the recombinant (TAL6003)-plasminogen, its N-terminus may be cleaved back (*e.g.,* cleaved back during activation) to provide a native-like N-terminus.

Generally, the invention provides recombinant (TAL6003)-plasmin(ogen) polypeptides having a single kringle region N-terminal to the activation site and serine protease domain, having certain advantages relative to mini-plasmin(ogen). Although the (TAL6003)-plasminogens of the invention only have one kringle domain, as such, N-terminal to the activation site, some embodiments include additional sequences N-terminal to the activation site. Additional N-terminal sequences can be derived from those of native kringle regions of plasminogen.

The N-terminal kringle domains of the present invention include kringle sequences of kringles 1 and 4 of native plasmin(ogen) and functional equivalents thereof. In particular, see the discussion below which provides guidance regarding preservation of function in polypeptide variants, including preservation of residues participating in or influencing lysine-binding.

Further, particular embodiments of the polypeptides of the present invention can exhibit reduced immunogenicity by virtue of native-like structures. For example, in some embodiments, the recombinant plasminogen of the present invention has an N-terminus identical to that of one of the naturally occurring forms of human plasma-derived plasminogen, which upon activation by streptokinase, produces plasmin polypeptides comprising native-like N-termini. Additionally, the novel polypeptides of the present invention have a sequence between the Kringle and Serine protease domains that is similar to the junction between Kringle 5 and the SP domain in naturally-occurring human plasmin.

Definitions

The terms "domain" and "region" of a polypeptide are generally synonymous as used herein, unless otherwise indicated to the contrary. When recited together with well-recognized structural or functional designations such as "kringle" or "serine protease," etc., such terms will introduce a polypeptide feature relating to at least some characteristic(s) commonly recognized and understood to be associated with the polypeptide structures corresponding to such designations.

A "cultured host cell," as used herein, refers to a prokaryotic or eukaryotic cell that contains heterologous DNA that has been introduced into the cell by any means, *e.g.*, electroporation, calcium phosphate precipitation, microinjection, transformation, viral infection, and the like.

"Heterologous" as used herein means "of different natural origin" or representing a non-natural state. For example, if a cultured host cell is transformed with a DNA or gene derived from another organism, particularly from another species, that gene is heterologous with respect to that cultured host cell and also with respect to descendants of the cultured host cell which carry that gene. Similarly, "heterologous" refers to a nucleotide sequence derived from and inserted into the same natural, original cell type, but which is present in a non-natural state, *e.g.*, a different copy number or under the control of different regulatory elements. Further, when used in the context of a nucleic acid or amino acid sequence, the term "heterologous" also may refer to any region of the sequence that is of a different natural origin than another region of the same sequence. For example, if a recombinant protein comprises a kringle domain derived from apolipoprotein(a) and a serine-protease domain derived from plasminogen, the kringle domain and the serine protease domain are "heterologous" relative to each other, particularly if each domain is derived from a different species or organism.

A "vector" molecule is a nucleic acid molecule into which heterologous nucleic acid can be inserted which can then be introduced into an appropriate cultured host cell. Vectors preferably have one or more origins of replication, and one or more sites into which the recombinant DNA can be inserted. Vectors often have convenient means by which cells with vectors can be selected from those without, *e.g.*, they encode drug resistance genes. Common vectors include plasmids, viral genomes, and (primarily in yeast and bacteria) "artificial chromosomes."

As used herein, the term "transcriptional control sequence" refers to nucleic acid sequences, such as initiator sequences, enhancer sequences and promoter sequences, which induce, repress, or otherwise control the transcription of protein encoding nucleic acid sequences to which they are operably-linked.

The term "polypeptide" is used interchangeably herein with the terms "peptide" and "protein."

The terms "polynucleotide" and "nucleic acid" are used interchangeably herein, and can refer to any nucleic acid that contains the information necessary for the purpose indicated by the context. That is, the nucleic acid can be DNA or RNA, either single stranded or double stranded, or other nucleic acid, as long as the polymer is capable of representing the appropriate information, *e.g.*, in relation to an encoded peptide, and can include complementary sequences, *e.g.*, sense strands and anti-sense strands of nucleic acids polymers.

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

The terms "N-terminal" and "C-terminal" are used herein to designate the relative position of any amino acid sequence or polypeptide domain or structure to which they are applied. The relative positioning will be apparent from the context. That is, an "N-terminal" feature will be located at least closer to the N-terminus of the polypeptide molecule than another feature discussed in the same context (the other feature possible referred to as "C-terminal" to the first feature). Similarly, the terms "5'-" and "3'-" can be used herein to designate relative positions of features of polynucleotides.

The polypeptides referred to herein as having an N-terminal domain "homologous to a kringle domain of native human plasminogen" exhibit structural and functional characteristics similar to native kringle domains of plasminogen.

Further, the polypeptides referred to herein as having an N-terminal domain "homologous to kringle 1" exhibit characteristics similar to native kringle 1, at least to the extent that the polypeptides can have a higher affinity for ω-aminocarboxylic acids (and functional homologs such as *trans*-4-aminomethylcyclohexane-1-carboxylic acid, a cyclic acid) than kringle 5. See, *e.g.*, Chang, Y., *et al.*, *Biochemistry 37*:3258-3271 (1998), incorporated herein by reference, for conditions and protocols for comparison of binding of isolated kringle domain polypeptides to 5-aminopentanoic acid (5-APnA); 6-aminohexanoic acid (6-AHxA), also known as ε-aminocaprioic acid (εACA); 7-aminoheptanoic acid (7-AHpA); and *trans*-4-aminomethylcyclohexane-1-carboxylic acid (t-AMCHA).

References to kringle domains "homologous to kringle 4" are defined similarly, as noted above regarding the phrase "homologous to kringle 1." That is, they exhibit functional characteristics similar to kringle 4 of native human plasminogen as discussed above. These polypeptides also bind immobilized lysine as described above.

The polypeptides of the invention bind immobilized lysine. As used herein, the phrase "binding immobilized lysine" means that the polypeptides so characterized are retarded in their progress relative to mini-plasminogen when subjected to column chromatography using lysine-SEPHAROSE as the chromatographic media. Typically, the polypeptides of the invention can be eluted from such chromatographic media (lysine affinity resins) using solutions containing the specific ligand, *e.g.*, εACA, as eluants.

Further, in addition to Chang *et al.*, *supra*, other references can be consulted by those of skill in the art to determine which residues can be varied by conservative or non-conservative substitution, deletion or addition to yield a deletion mutant within the scope of the present invention. For example, the following references provide information regarding particular residues of the native kringle domains that may be important for binding of ω aminocarboxylic acids: U.S. Pat. No. 6,538,103 to Ji, *et al.*; U.S. Pat. No. 6,218,517 to Suzuki; Douglas, J.T., *et al.*, *Biochemistry 41(10)*:3302-10 (2002); Zajicek, J., *et al.*, *J. Mol. Biol., 301(2)*:333-47 (2000); Lee, H., *et al.*, *Arch Biochem Biophys., 375(2)*:359-63 (2000); Castellino, F. and S. McCance, *Ciba Found Symp. 212*:46-60 (1997); McCance, S., *et al.*, J. Biol. Chem., *269*:32405-32410 (1994); Rejante, M.R. and M. Llinas, *Eur. J. Biochem., 221(3)*:939-

49 (1994); Wu, T.P., *et al.*, *Blood Coagul. Fibrinolysis*, *5(2)*:157-66 (1994); Hoover, C.J., *et al.*, *Biochemistry*, *32(41)*:10936-43 (1993); Menhart, N., *et al.*, *Biochemistry*, *32*:8799-8806 (1993); Thewes, T., *et al.*, *J. Biol. Chem.*, *265 (7)*:3906-3915 (1990); Novokhatny, V., *et al.*, *Thromb Res.*, *53(3)*:243-52 (1989); Motta, A., *et al.*, *Biochemistry*, *26(13)*:3827-36 (1987); Novokhatny, V., *et al.*, *J. Mol. Biol.*, *179*:215-232 (1984); Lerch, P.G., *et al.*, *Eur. J. Biochem.*, *107(1)*:7-13 (1980); Sottrup-Jensen, L., *et al.*, *Prog. Chem. Fibrinol. Thrombol.*, *3*:191-209 (1978); and Wiman, B. and D. Collen, *Nature 272*, 549-545 (1978), all incorporated herein by reference in their entirety.

Because the present inventors have recognized that a valuable, simplified plasmin(ogen) molecule can be prepared having a single N-terminal kringle domain having advantageous functional characteristics (which can be evaluated, in part, by testing for the binding of immobilized lysine as described herein), the present invention can encompass other fibrin-binding domains or regions N-terminal to the activation site. For example, the invention can include polypeptides in which the serine protease domain of plasmin is attached to a fibrin-binding kringle selected from a group including, but not limited to, kringle 4 of human plasminogen, kringle 2 of tPA, or a kringle of apolipoprotein (a). Further, the invention can include polypeptides in which a serine protease domain of plasmin is attached to any other known fibrin-binding modules, such as the "finger" domain of tPA or fibronectin, or the FAB fragment of fibrin-specific IgG.

In some aspects, the polypeptides of the present invention have protein chemical structures (*e.g.,* native-like N-terminus and native-like juncture between the kringle and the serine protease domain) that are identical to the chemical structures found in the naturally occurring forms of human plasma-derived plasmin(ogen). Without being held to a particular theory, it is believed that certain features of a protein can contribute to its immunogenicity, including but not limited to its amino acid sequence. Accordingly, the present invention provides an effective protein therapeutic based on recombinant (TAL6003)-plasminogen by pre-emptively reducing the potential immunogenicity of (TAL6003)-plasminogen through incorporation of amino acid sequences that resemble native human plasminogen sequences.

In one aspect, the recombinant (TAL6003)-plasminogen polypeptide of the present invention comprises a) a single N-terminal kringle domain homologous to a kringle domain of native human plasminogen, wherein the last four amino acid residues within the kringle domain are V,P,Q, and C; and b) a C-terminal domain activation site and serine protease domain homologous to the corresponding domains in human plasminogen; wherein the polypeptide binds to immobilized lysine. In one embodiment, the single N-terminal kringle domain is homologous to kringle 1 or kringle 4 of native human plasminogen. In some embodiments, the immobilized lysine is lysine bound to a solid support matrix selected from the group consisting of lysine-agarose, lysine-hydrogel, lysine-cross-linked agarose. In another embodiment, the immobilized lysine is lysine-cross-linked agarose.

The recombinant (TAL6003)-plasminogen polypeptides of the present invention can be activated by one of ordinary skill in the art to provide a (TAL6003)-plasmin polypeptide. In one embodiment, the (TAL6003)-plasmin polypeptide exhibits a fibrinolytic activity that is inhibited by α_2-antiplasmin at a rate of inhibition that is at least about 5-fold faster than the rate of inhibition of the fibrinolytic activity of mini-plasmin by α_2-antiplasmin. In another embodiment, the rate of inhibition is at least about 10-fold, 20-fold, 30-fold, or 40-fold faster than the rate of inhibition of mini-plasmin.

In one embodiment, the recombinant (TAL6003)-plasminogen polypeptide is at least 90% or 95%, or 98% identical to the sequence shown in SEQ ID NO: 2. In another embodiment, the single N-terminal kringle domain is at least 90% identical to the kringle 1 or kringle 4 domain of native human plasminogen; and the C-terminal domain is at least 90% identical to the activation site and serine protease domain of human plasminogen. In some embodiments, the polypeptide has an amino acid sequence as shown in SEQ ID NO: 2, and conservative substitutions thereof. In other embodiments, the polypeptide has an arginine residue at a relative position analogous to that of position 85 of the amino acid sequence shown in SEQ ID NO: 2.

In further embodiments, the single N-terminal kringle domain has at least one residue greater amino acid sequence identity with kringle 1 or kringle 4 of native human plasminogen than with kringle 5 of native human plasminogen, and wherein conservative substitutions of the single N-terminal kringle domain relative to the native sequences of kringles 1 and 4 of human plasminogen are not considered as

differing from the native sequences for purposes of the identity comparison with kringle 5.

For example, the (TAL6003)-plasminogen described in this invention makes use of amino acid residue modifications to the junction region joining the single kringle domain and the serine protease domain. Accordingly, this juncture between the two domains more closely resembles the naturally occurring juncture between the kringle 5 domain and the serine protease domain of human plasminogen.

In another embodiment, the (TAL6003)-plasminogen described in this invention further comprises a native-like N-terminal sequence. The recombinantly produced (TAL6003)-plasminogen can be cleaved off upon activation to provide recombinant (TAL6003)-plasmin polypeptides also having native-like N-termini.

In particular embodiments, residues at certain positions of the single N-terminal kringle domain of (TAL6003)-plasminogen are conserved relative to kringle 1 of native human plasminogen. These can be residues at positions associated with disulfide bridging and lysine binding, and include Cys84, Cys105, Cys133, Cys145, Cys157, and Cys162, and Pro136-Pro140, Pro143-Tyr146, and Arg153-Tyr156, respectively (positions numbered as shown in Figure 3). Additionally, particular embodiments of the invention can be characterized chemically by contrast to mini-plasmin(ogen) which has an analogous domain composition (*i.e.*, kringle-serine protease (K-SP) (see Sottrup-Jensen, L., *et al.*, Progress in Chemical Fibrinolysis and Thrombolysis, Vol. 3, (Eds: J. F. Davidson, *et al.*) Raven Press, New York (1978)) but, inter alia, lacks an arginine (Arg) at a relative position analogous to that of position 85 of the amino acid sequence shown in SEQ ID NO: 2. In some embodiments, the (TAL6003)-plasminogen of the invention comprises a single N-terminal kringle domain comprising an Arg residue at a relative position analogous to that of position 85 of the amino acid sequence shown in SEQ ID NO: 2. Non-limiting examples of a relative position analogous to that of position 85 of the amino acid sequence shown in SEQ ID NO: 2 include Arg(153), Arg(234), Arg(324), and Arg(426) positions of the amino acid sequence shown in SEQ ID NO:4.

In other embodiments, the specific positions of the named residues can vary somewhat while still being present in the polypeptide at structurally and functionally analogous positions (*i.e.* relative to the kringle structure of the N-terminal domain; see Chang, Y., *et al.* as discussed above). In some embodiments, the single N-terminal

kringle domain of the (TAL6003)-plasmin(ogen) polypeptide has at least one residue greater percent identity with kringle 1 or kringle 4 of native human plasminogen than with kringle 5 of native human plasminogen.

Further, particular embodiments of the invention can be characterized functionally by contrast to mini-plasmin(ogen). In preferred embodiments, the (TAL6003)-plasmin of the invention exhibits an increased rate of inhibition by α_2-antiplasmin, *e.g.*, as much as about one or two orders of magnitude faster than the rate of inhibition of mini-plasmin. Further, in particular embodiments, (TAL6003)-plasmin binds immobilized lysine (*e.g.*, lysine-SEPHAROSE).

Characterization of the single N-terminal kringle domain of (TAL6003)-plasminogen as "N-terminal" means only that the domain is present N-terminal to the activation site and does not mean that additional amino acids residues N-terminal to the domain itself are not present. Further, the number and identity of residues interposed between the most C-terminal cysteine residue of the single N-terminal kringle domain (*i.e.*, the most C-terminal Cys residue shown in Figure 4) and the activation site of plasminogen can be varied without departing from the scope of the present invention. One of skill in the art will be able to determine these variations that achieve the benefits of the invention (kringle 1-like binding of ω aminocarboxylic acids, without substantial increase in size of the deletion mutant or introduction of potentially problematic glycosylation sites) without undue experimentation based on the disclosure herein and the references cited herein for guidance regarding kringle 1 function and structure.

Accordingly, the invention relates to polynucleotides, polypeptides, recombinant methods for producing the polypeptides, vectors containing the polynucleotides, expression systems for producing the polypeptides, and cultured host cells comprising such expression systems.

As noted, in one aspect, the invention relates to a polynucleotide encoding the polypeptide disclosed herein or a polypeptide having conservative amino acid substitutions thereof. Guidance regarding selection of "conservative" amino acid substitutions is provided in more detail below. In one embodiment, the polynucleotide is DNA.

In another aspect, the invention relates to a method of making a vector comprising inserting the polynucleotide of the invention into a vector. In another aspect, the invention relates to a vector produced by the method of the invention.

In another aspect, the invention relates to a method of making a cultured host cell comprising introducing the vector of the invention into a cultured host cell. In another aspect, the invention relates to a cultured host cell produced by the method of the invention.

In another aspect, the invention relates to an isolated polypeptide of the invention, produced by a method comprising: (a) introducing a vector comprising a polynucleotide encoding the polypeptide into a cultured host cell; (b) culturing the host cell; and (c) recovering the polypeptide. In another aspect, the invention relates to a method for producing a polypeptide comprising: (a) culturing the host cell of the invention under conditions that the vector is expressed; and (b) recovering the polypeptide.

In another aspect, the invention relates to cells containing at least one polynucleotide of the invention.

In one embodiment, the polynucleotide comprises the nucleotide sequence as shown in SEQ ID NO:1. In another embodiment, the polynucleotide comprises nucleotides 4 through 1032 of the nucleotide sequence shown in SEQ ID NO:1. In other embodiments, the polypeptide comprises the amino acid sequence as shown in SEQ ID NO:2.

Polynucleotides

The polynucleotides of the invention include variants which have substitutions, deletions, and/or additions which can involve one or more nucleotides. The variants can be altered in coding regions, non-coding regions, or both. Alterations in the coding regions can produce conservative or non-conservative amino acid substitutions, deletions or additions. Especially preferred among these are silent substitutions, additions and deletions, which do not alter the properties and activities of the (TAL6003)-plasmin(ogen) protein or portions thereof. Also especially preferred in this regard are conservative substitutions (see below).

Further embodiments of the invention include nucleic acid molecules comprising a polynucleotide having a nucleotide sequence at least 90% identical, and more preferably at least 95%, 96%, 97%, 98% or 99% identical to (a) a nucleotide

sequence encoding a polypeptide having the complete amino acid sequence in SEQ ID NO:2; (b) a nucleotide sequence encoding a polypeptide having the amino acid sequence in SEQ ID NO:2; and (c) a nucleotide sequence complementary to any of the nucleotide sequences in (a) or (b) above.

In one embodiment, the nucleic acid molecule of the present invention comprises a polynucleotide having a nucleotide sequence that encodes a polypeptide having the amino acid sequence shown in SEQ ID NO: 2. In other embodiments, the nucleic acid molecule comprises a polynucleotide having the nucleotide sequence shown in SEQ ID NO:1, or a degenerate variant thereof.

By a polynucleotide having a nucleotide sequence at least, for example, 95% "identical" to a reference nucleotide sequence encoding a (TAL6003)-plasminogen is intended that the nucleotide sequence of the polynucleotide be identical to the reference sequence except that the polynucleotide sequence can include up to five point mutations per each 100 nucleotides of the reference nucleotide sequence encoding the (TAL6003)-plasminogen polypeptide. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence can be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence can be inserted into the reference sequence. These mutations of the reference sequence can occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence.

As noted above, two or more polynucleotide sequences can be compared by determining their percent identity. Two or more amino acid sequences likewise can be compared by determining their percent identity. The percent identity of two sequences, whether nucleic acid or peptide sequences, is generally described as the number of exact matches between two aligned sequences divided by the length of the shorter sequence and multiplied by 100. An approximate alignment for nucleic acid sequences is provided by the local homology algorithm of Smith and Waterman, Advances in Applied Mathematics 2:482-489 (1981). This algorithm can be extended to use with peptide sequences using the scoring matrix developed by Dayhoff, Atlas of Protein Sequences and Structure, M. O. Dayhoff ed., 5 suppl. 3:353-358, National

Biomedical Research Foundation, Washington, D.C., USA, and normalized by Gribskov, *Nucl. Acids Res. 14(6)*:6745-6763 (1986). An implementation of this algorithm for nucleic acid and peptide sequences is provided by the Genetics Computer Group (Madison, Wis.) in their BESTFIT utility application. The default parameters for this method are described in the Wisconsin Sequence Analysis Package Program Manual, Version 8 (1995) (available from Genetics Computer Group, Madison, Wis.).

For example, due to the degeneracy of the genetic code, one of ordinary skill in the art will immediately recognize that a large number of the nucleic acid molecules having a sequence at least 90%, 95%, 96%, 97%, 98%, or 99% identical to the nucleic acid sequence shown in SEQ ID NO:1 will encode a (TAL6003)-plasminogen polypeptide. In fact, because degenerate variants of these nucleotide sequences all encode the same polypeptide, this will be clear to the skilled artisan even without performing any functional assays or measurements described herein. It will be further recognized in the art that, for such nucleic acid molecules that are not degenerate variants, a reasonable number will also encode a polypeptide having (TAL6003)-plasminogen polypeptide activity. This is because the skilled artisan is fully aware of amino acid substitutions that are either less likely or not likely to significantly effect protein function (*e.g.*, replacing one aliphatic amino acid with a second aliphatic amino acid).

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

Of course, traditional techniques of molecular biology, microbiology, and recombinant nucleic acid can also be used to produce the polynucleotides of the invention. These techniques are well known and are explained in, for example,

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

Vectors and Cultured Host Cells

The present invention also relates to vectors which include the isolated nucleic acid molecules of the present invention, cultured host cells which are genetically engineered with the recombinant vectors, and the production of the (TAL6003)-plasmin(ogen) polypeptides by recombinant techniques.

Recombinant constructs can be introduced into cultured host cells using well-known techniques such as infection, transduction, transfection, transvection, electroporation and transformation. The vector can be, for example, a phage, plasmid, viral or retroviral vector. Retroviral vectors can be replication competent or replication defective. In the latter case, viral propagation generally will occur only in complementing cultured host cells.

The polynucleotides can be joined to a vector containing a selectable marker for propagation in a cultured host. Generally, a plasmid vector is introduced in a precipitate, such as a calcium phosphate precipitate, or in a complex with a charged lipid. If the vector is a virus, it can be packaged *in vitro* using an appropriate packaging cell line and then transduced into cultured host cells.

Preferred are vectors comprising *cis*-acting control regions to the polynucleotide of interest. Appropriate *trans*-acting factors can be supplied by the cultured host, supplied by a complementing vector or supplied by the vector itself upon introduction into the cultured host.

In certain embodiments in this regard, the vectors provide for specific expression, which can be inducible and/or cell type-specific. Particularly preferred among such vectors are those inducible by environmental factors that are easy to manipulate, such as temperature and nutrient additives.

Expression vectors useful in the present invention include chromosomal-, episomal- and virus-derived vectors, *e.g.*, vectors derived from bacterial plasmids, bacteriophage, yeast episomes, yeast chromosomal elements, viruses such as baculoviruses, papova viruses, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as cosmids and phagemids.

DNA inserts should be operatively linked to an appropriate promoter, such as the phage lambda PL promoter, the *E. coli* lac, trp and tac promoters, the SV40 early and late promoters and promoters of retroviral LTRs, to name a few. Other suitable promoters will be known to the skilled artisan. The expression constructs will further contain sites for transcription initiation, termination and, in the transcribed region, a ribosome binding site for translation. The coding portion of the mature transcripts expressed by the constructs can include a translation initiating at the beginning and a termination codon (UAA, UGA or UAG) appropriately positioned at the end of the polypeptide to be translated.

As indicated, the expression vectors will preferably include at least one selectable marker. Such markers include dihydrofolate reductase or neomycin resistance for eukaryotic cell culture and tetracycline or ampicillin resistance genes for culturing in *E. coli* and other bacteria. Representative examples of appropriate cultured hosts include, but are not limited to, bacterial cells, such as *E. coli*, *Streptomyces* and *Salmonella typhimurium* cells; fungal cells, such as yeast cells; insect cells such as *Drosophila* S2 and *Spodoptera* Sf9 cells; animal cells such as CHO, COS and Bowes melanoma cells; and plant cells. Appropriate culture mediums and conditions for the above-described cultured host cells are known in the art.

Among vectors preferred for use in bacteria include e.g., pET24b or pET22b available from Novagen, Madison, WI (pET-24b(+) and pET-22b(+) = pET Expression System 24b (Cat. No. 69750) and 22b (Cat. No. 70765), respectively, EMD Biosciences, Inc., Novagen Brand, Madison, WI; see http://www.emdbiosciences.com product information section regarding pET-24b and

pET-22b for details regarding vector), pQE70, pQE60 and pQE-9, available from Qiagen Inc., Valencia, CA; pBS vectors, PHAGESCRIPT vectors, BLUESCRIPT vectors, pNH8A, pNH16a, pNH18A, pNH46A, available from Stratagene, LaJolla, CA; and ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia (now Pfizer, Inc., New York, NY). Among preferred eukaryotic vectors are pWLNEO, pSV2CAT, pOG44, pXT1 and pSG available from Stratagene; and pSVK3, pBPV, pMSG and pSVL available from Pharmacia. Other suitable vectors will be readily apparent to the skilled artisan.

Bacterial promoters suitable for use in the present invention include the *E. coli* lacI and lacZ promoters, the T3 and T7 promoters, the gpt promoter, the lambda PR and PL promoters, and the trp promoter. Suitable eukaryotic promoters include the CMV immediate early promoter, the HSV thymidine kinase promoter, the early and late SV40 promoters, the promoters of retroviral LTRs, such as those of the Rous sarcoma virus (RSV), and metallothionein promoters, such as the mouse metallothionein-I promoter.

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

Transcription of the DNA encoding the polypeptides of the present invention by higher eukaryotes can be increased by inserting an enhancer sequence into the vector. Enhancers are *cis*-acting elements of DNA, usually about from 10 to 300 bp that act to increase transcriptional activity of a promoter in a given cultured host cell-type. Examples of enhancers include the SV40 enhancer, which is located on the late side of the replication origin at bp 100 to 270, the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

For secretion of the translated protein into the lumen of the endoplasmic reticulum, into the periplasmic space or into the extracellular environment, appropriate secretion signals can be incorporated into the expressed polypeptide. The signals can be endogenous to the polypeptide or they can be heterologous signals.

The polypeptide can be expressed in a modified form, such as a fusion protein, and can include not only secretion signals, but also additional heterologous functional regions. For instance, a region of additional amino acids, particularly charged amino acids, can be added to the N-terminus, for example, the polypeptide to improve stability and persistence in the cultured host cell, during purification, or during subsequent handling and storage. Also, peptide moieties can be added to the polypeptide to facilitate purification. Such regions can be removed prior to final preparation of the polypeptide. The addition of peptide moieties to polypeptides to engender secretion or excretion, to improve stability and to facilitate purification, among others, are familiar and routine techniques in the art. A preferred fusion protein comprises a heterologous region from immunoglobulin that is useful to solubilize proteins. For example, EP 0 464 533 A1 (Canadian counterpart, 2,045,869) discloses fusion proteins comprising various portions of constant region of immunoglobulin molecules together with another human protein or part thereof. In many cases, the Fc part in a fusion protein is thoroughly advantageous for use in therapy and diagnosis and thus results, for example, in improved pharmacokinetic properties. On the other hand, for some uses it would be desirable to be able to delete the Fc part after the fusion protein has been expressed, detected and purified in the advantageous manner described. This is the case when Fc portion proves to be a hindrance to use in therapy and diagnosis, for example when the fusion protein is to be used as antigen for immunizations. In drug discovery for example, human proteins have been fused with Fc portions for the purpose of high-throughput screening assays (such as hIL5-receptor, to identify antagonists of hIL-5). See, Bennett, D., *et al.*, *J. Molecular Recognition*, *8*:52-58(1995) and Johanson, K. *et al.*, *J. Biol.Chem.*, *270(16)*:9459-9471 (1995).

(TAL6003)-plasminogen can be recovered and purified from recombinant cell cultures by well-known methods including those specifically described in the examples herein. Polypeptides of the present invention include naturally purified products, products of chemical synthetic procedures, and products produced by recombinant techniques from a prokaryotic or eukaryotic cultured host, including, for example, bacterial, yeast, higher plant, insect and mammalian cells. In addition, polypeptides of the invention can also include an initial modified methionine residue, in some cases as a result of host-mediated processes.

Polypeptides

The polynucleotides of the invention include those encoding variations and particular examples of the polypeptides of the invention. For example, guidance concerning how to make phenotypically silent amino acid substitutions is provided in Bowie, J. U. *et al.*, "Deciphering the Message in Protein Sequences: Tolerance to Amino Acid Substitutions," *Science 247*:1306-1310 (1990), wherein the authors indicate that proteins are surprisingly tolerant of amino acid substitutions. Although any number of substitutions within the scope of the invention can be obtained by application of such general principles, for specific guidance regarding substitutions, the references cited herein regarding structure and function of kringle 1 domains can be consulted by one of skill in the art.

It will further be appreciated that, depending on the criteria used, the exact "position" or sequence of the kringle, activation site, and serine protease domains of the (TAL6003)-plasminogen can differ slightly in particular variations within the scope of the present invention. For example, the exact location of the kringle domain relative to the activation site can vary slightly and/or the sequence N-terminal to the kringle domain can vary in length. Thus, the invention includes such variations of the (TAL6003)-plasminogen polypeptides which exhibit (TAL6003)-plasminogen polypeptide activity as disclosed herein. Such variants include deletions, insertions, inversions, repeats, and substitutions. As indicated above, guidance concerning which amino acid changes are likely to be phenotypically silent can be found in Bowie, J. U., *et al.*, "Deciphering the Message in Protein Sequences: Tolerance to Amino Acid Substitutions," *Science 247*:1306-1310 (1990).

Thus, fragments, derivatives or analogs of the polypeptide of SEQ ID NO:2 can be (i) ones in which one or more of the amino acid residues (*e.g.*, 3, 5, 8, 10, 15 or 20 residues) are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue). Such substituted amino acid residues may or may not be one encoded by the genetic code, or (ii) ones in which one or more of the amino acid residues includes a substituent group (*e.g.*, 3, 5, 8, 10, 15 or 20), or (iii) ones in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or (iv) ones in which the additional amino acids are fused to the mature polypeptide, such as an IgG Fc fusion region peptide or leader or secretory sequence

or a sequence which is employed for purification of the mature polypeptide or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

As indicated, changes are preferably of a minor nature, such as conservative amino acid substitutions that do not significantly affect the folding or activity of the protein. Of course, the number of amino acid substitutions a skilled artisan would make depends on many factors, including those described above. Generally speaking, the number of substitutions for any given (TAL6003)-plasminogen polypeptide will not be more than 50, 40, 30, 25, 20, 15, 10, 5 or 3.

Amino acids in the (TAL6003)-plasminogen of the present invention that are essential for function can be identified by methods known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, *Science 244*:1081-1085 (1989)). The latter procedure introduces single alanine mutations at every residue in the molecule. The resulting mutant molecules are then tested for biological activity, *e.g.*, as shown in the examples provided herein. Sites that are critical for ligand binding can also be determined by structural analysis such as crystallization, nuclear magnetic resonance or photoaffinity labeling (Smith, *et al.*, *J. Mol. Biol. 224*:399-904 (1992) and de Vos, *et al. Science 255*:306-312 (1992)). Even if deletion of one or more amino acids from the N-terminus of a protein results in modification or loss of one or more biological functions of the protein, other biological activities can still be retained.

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

The polypeptides of the present invention can be provided in an isolated form. By "isolated polypeptide" is intended a polypeptide removed from its native environment. Thus, a polypeptide produced and/or contained within a recombinant cultured host cell is considered isolated for purposes of the present invention. Also intended as an "isolated polypeptide" are polypeptides that have been purified, partially or substantially, from a recombinant cultured host.

Polypeptides having an amino acid sequence of an indicated percent identity to a reference amino acid sequence of a (TAL6003)-plasminogen polypeptide can be

determined using the methods, including computer-assisted methods, indicated above regarding polynucleotides. Polypeptide amino acid sequences are examined and compared just as are the nucleotide sequences in the foregoing discussion. One of skill in the art will recognize that such concepts as the molecular endpoints discussed for polynucleotides will have direct analogs when considering the corresponding use of such methods and programs for polypeptide analysis. For example, the manual corrections discussed regarding polynucleotides refer to 5' and 3' endpoints of nucleic acids, but the same discussion will be recognized as applicable to N-termini and C-termini of polypeptides.

The invention encompasses (TAL6003)-plasminogen polypeptides which are differentially modified during or after translation, *e.g.*, by glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, *etc.* Any of numerous chemical modifications can be carried out by known techniques, including but not limited, to specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, *S. aureus* V8 protease, $NaBH_4$; acetylation, formylation, oxidation, reduction; metabolic synthesis in the presence of tunicamycin; *etc.*

Additional post-translational modifications encompassed by the invention include, for example, *e.g.*, N-linked or O-linked carbohydrate chains, processing of N-terminal or C-terminal ends, attachment of chemical moieties to the amino acid backbone, chemical modifications of N-linked or O-linked carbohydrate chains, and addition of an N-terminal methionine residue as a result of vectors and constructs adapted for expression of (TAL6003)-plasminogen polypeptides in prokaryotic cultured host cells. The polypeptides can also be modified with a detectable label, such as an enzymatic, fluorescent, isotopic or affinity label to allow for detection and isolation of the protein.

Pharmaceutical Compositions and Methods of Treatment

(TAL6003)-plasmin(ogen) can be formulated for therapeutic use in accordance with the methods and compositions described in U.S. Patent Application No. 10/143,112; and Novokhatny, V., *et al.*, *J. Thromb. Haemost. 1(5)*:1034-41 (2003), both incorporated herein by reference. For example, a low-pH (from about 2.5 to about 4), low-buffering capacity buffer can be used for formulation of (TAL6003)-plasmin. Additionally, other methods and formulations known to those of skill in the art, as practiced with plasmin, mini-plasmin, and/or micro-plasmin, can be used to formulate the (TAL6003)-plasmin of the invention for therapeutic administration.

The (TAL6003)-plasmin(ogen) can be used to treat a variety of thrombotic diseases or conditions, for example, according to the methods as described in U.S. Patent Nos. 6,355,243, 6,964,764, and 6,969,515, all incorporated herein by reference. Again, as with the possible pharmaceutical formulations applicable to (TAL6003)-plasmin, (TAL6003)-plasmin can also be administered therapeutically by methods known in the art, for example, those that may be currently practiced with plasmin, mini-plasmin, and/or micro-plasmin.

EXAMPLES

Expression Vector Design

The amino acid sequence for (TAL6003)-plasminogen is shown in SEQ ID NO: 2. A polynucleotide having the nucleotide sequence encoding (TAL6003)-plasminogen was codon-optimized for *E. coli* expression and mRNA stability to provide the DNA sequence as shown in SEQ ID NO:1. This polynucleotide was cloned into the NdeI and BamH1 sites of *E. coli* expression vector pET24b(+) (Novagen; Madison, WI) to produce cytosolic protein.

As illustrated in Table 1, expression in bacteria (*e.g., E. coli*) provided a recombinant (TAL6003)-plasminogen polypeptide having the amino acid sequence as shown in SEQ ID NO:2 (*i.e.*, a recombinant (TAL6003)-plasminogen with an N-terminal methionine (*i.e.*, M^1) immediately preceding the arginine amino acid residue (*i.e.*, R^2) corresponding to the arginine at position 70 (*i.e.*, R^{70}) of the native human plasminogen amino acid sequence shown in SEQ ID NO:4 (see also, *e.g.*, Fig. 3). Such a recombinant product was susceptible to further cleavage to yield additional

proteins having different N-termini including a protein with an N-terminal lysine (*i.e.*, K^{10}) or valine (*i.e.*, V^{11}) corresponding, respectively, to the lysine at position 78 (*i.e.*, K^{78}) or the valine at position 79 (*i.e.*, V^{79}) of native human plasminogen.

Table 1: N-termini of native plasmin(ogen) (*e.g.*, based on SEQ ID NO:4) and (TAL6003)-plasmin(ogen) (*e.g.*, based on SEQ ID NO:2, or a variant thereof)

Native Plasminogen comprising 19 amino acid leader sequence (*e.g.*, based on SEQ ID NO:4): ↓ ↓ ↓ ↓ $M^{-19}EHKE$ ... $E^{01}PLDDY$ ... $M^{69}R^{70}DVVLFEKK^{78}V^{79}YLSEC$ .....
Native "Lys-Plasminogen" (*i.e.*, cleavage of leader sequence): ↓ ↓ ↓ $E^{01}PLDDY$ ... $M^{69}R^{70}DVVLFEKK^{78}V^{79}YLSEC$ ..... (SEQ ID NO:5)
Native Plasmin species possible based on cleavage, if any, of Lys-Plasminogen: (SEQ ID NO:6) $M^{69}R^{70}DVVLFEKK^{78}V^{79}YLSEC$ ..... (SEQ ID NO:7) $K^{78}V^{79}YLSEC$ ..... (SEQ ID NO:8) $V^{79}YLSEC$ .....
Recombinant (TAL6003)-plasminogen polypeptides of the present invention: ↓ ↓ (*e.g.*, based on SEQ ID NO:2) $M^{01}R^{02}DVVLFEKK^{10}V^{11}YLSEC$ .....
Additional proteins based on further cleavage of a (TAL6003)-plasminogen: (SEQ ID NO:9) $K^{10}V^{11}YLSEC$ ..... (SEQ ID NO:10) $V^{11}YLSEC$ .....

↓ indicates potential cleavage sites.

(TAL6003)-plasminogen Expression and Purification

The expression vector comprising the DNA encoding (TAL6003)-plasminogen was transformed into a variety of cells including BL21(DE3) RIL (Stratagene, La Jolla, CA), BL21(DE3) (genotype: F⁻*ompT* *hsd*S_B ($r_B^-m_B^-$) *gal dcm* (DE3)) (EMB Biosciences, Inc., San Diego, CA), and BLR(DE3) (genotype: F⁻*ompT* *hsd*S_B ($r_B^-m_B^-$) *gal dcm* (DE3) Δ(*srl-recA*)*306*::Tn*10*(Tet^R)), and protein over-expression following induction by 1mM IPTG (isopropyl-beta-D-thiogalactopyranoside) was analyzed by SDS-PAGE. Expression estimates were at least about 250 mg/L cell culture in shaker flasks.

Cell type BL21(DE3) RIL is engineered to express rare *E. coli* tRNAs coding for Arg, Ile, and Leu. Further, both BL21(DE3) and BLR(DE3) are B strain *E. coli* that is classified as non-pathogenic to humans and animals based on the absence of virulence and colonization factors. BLR(DE3) cells lack the recA gene for DNA recombination, and induction of lamba phage has not been reported with these cells.

A research cell bank of the (TAL6003)-plasminogen construct in BLR(DE3) cells was produced and tested for purity, identity, and induction of bacteriophage at Charles River Laboratories (Malvern, PA). The testing confirmed the identity and purity of the research cell bank and the cells passed the phage induction test with no phage observed (data not shown).

Production of (TAL6003)-plasminogen (*i.e.,* based on SEQ ID NO:2) was confirmed in larger scale expression in which cells were lysed and both soluble protein and purified inclusion bodies were examined by SDS-PAGE.

The following typical protocol has been used for expression of (TAL6003)-plasminogen:

A single colony of *E. coli* cells (*e.g.,* BL21(DE3) RIL, BL21(DE3), or BLR(DE3) containing the (TAL6003)-plasminogen vector was used to inoculate 5 ml of LB/kanamycin (30 μg/ml) and was incubated for 8 hours at 37°C on a shaker. After that, a 50 μl-aliquot was taken form the cultured bacterial suspension for further growth in fresh media. The procedure was repeated after 16 hours with 6 ml of bacterial culture and 250 ml of the media. Cultures were grown at 37°C with shaking to an OD600 nm of ~ 1.0, and IPTG was added to 1 mM final concentration. Cultures were grown for an additional 5 hours. Cells were harvested by centrifugation at 5,000 x g and cell pellets were dissolved in 20 mM Tris pH 8.0 containing 20 mM EDTA and frozen at –80 °C.

To purify (TAL6003)-plasminogen, cell pellets were thawed and buffer added until the solution volume was approximately 1/20th that of the original cell culture volume. After that, lysozyme was added to a final concentration of 0.5 mg/ml and the cells were stirred rapidly at 4°C for 10 - 15 minute. Then, Triton X-100 was added to 1% final concentration and stirring continued for another 10 min. DNAse I (0.05 mg/ml) and $MgCl_2$ (2.5 mM) were added and stirring was continued at 4°C for 30 minutes or until the solution was no longer viscous. The final solution was centrifuged at 4°C for 30 min at 15,000 x g and the supernatant was discarded.

The cell pellet was washed three times with wash solution (50 mM Tris-HCl, pH 7.4 containing 10 mM EDTA, 1% Triton-X-100, and 0.5 M urea), and the final pellet was dissolved in 40 ml of extraction buffer (PBS, pH 7.4 containing 10 mM EDTA, 20 mM DTT, and 6 M guanidine-HCl) and stored at 4 °C overnight. After 16 hours, the solution was centrifuged for 30 minutes at 15,000 x g to remove solids and

the supernatant was slowly added to the refolding solution (50 mM Tris-HCl, pH 8.3, 3.5 M guanidine HCl, 0.5 M arginine HCl, 10 mM EDTA, 3 mM GSH, 0.3 mM GSSG) while stirring at 4°C. The refolding procedure was carried out at protein concentration of about 0.29 g/L.

The refolding solution was kept for 2 days at 4°C undisturbed and then dialyzed against an 8-fold volume of 0.1 M Tris-HCl pH 8.0 containing 10 mM EDTA, 0.15 M NaCl, 0.15 M arginine-HCl, over a period of 8-10 hours with frequent changes of the buffer solution.

The protein solution was then removed from dialysis and concentrated using AMICON filters with the membrane cut-off of 10 kDa to approximately 10–20 ml and dialyzed overnight versus a 100-fold volume of 0.1 M Tris pH 8.0 containing 10 mM EDTA, 0.15 M NaCl. This material was centrifuged to remove particulates, then passed over lysine affinity resin (Lysine-SEPHAROSE 4B; Amersham Biosciences, Piscataway, NJ). (TAL6003)-plasminogen was eluted from the resin using Tris-buffered saline, pH 8.0 containing 0.2 M epsilon aminocaproic acid (εACA).

Typically, 80 mg of inclusion bodies could be isolated from 1 liter of cell culture and 40 mg could be eluted in the lysine-SEPHAROSE chromatography step.

Properties of (TAL6003)-plasminogen

Purified (TAL6003)-plasminogen appeared as a band in the 35-40 kDa region by SDS-PAGE analysis of reduced (dithiothreitol-treated) and non-reduced protein. Its molecular mass, determined by MALDI mass-spectrometry, was about 38,140 Da, which is close to the expected value.

To determine the rate of activation of (TAL6003)-plasminogen by streptokinase, 1 mg/ml of recombinant (TAL6003)-plasminogen was mixed with streptokinase at a 1:100 (TAL6003)-plasminogen to streptokinase ratio and incubated at room temperature at pH 7. At various time points, samples were removed and quenched with SDS-Page buffer and analyzed on reduced SDS-PAGE followed by densitometry to determine the conversion of the one-chain (TAL6003)-plasminogen molecule into a two-chain (TAL6003)-plasmin. Percent activation of (TAL6003)-plasminogen by streptokinase is shown in Figure 6 as loss of full-length (TAL6003)-plasminogen over time as determined by SDS-PAGE.

To confirm the functionality of kringle 1, we determined the binding of TAL6003-plasminogen to lysine-SEPHAROSE 4B. As shown in Figure 7,

(TAL6003)-plasminogen bound to lysine-SEPHAROSE and could be eluted from the column by a 0-20 mM εACA gradient as a single peak at about 4 mM. The ability of refolded (TAL6003)-plasminogen to bind lysine-SEPHAROSE indicates that the kringle domain of the molecule is properly folded and the lysine-binding site is fully active.

To further confirm the functionality of kringle 1, the binding of εACA to (TAL6003)-plasminogen was measured by monitoring the associated changes in protein fluorescence as described by Matsuka *et al.*, Eur. J. Biochem., 190:93-97 (1990) and Douglas *et al.,* J. Biochemistry 41:3302-3310 (2002), all incorporated herein by reference. Binding of εACA to kringle 1 of (TAL6003)-plasminogen results in a decrease in fluorescence, likely due to quenching of the tryptophan residues which are part of the lysine-binding site.

To monitor this process, 4 µl to 16 µl aliquots of a concentrated solution of εACA were added to 2 ml of 5 µM (TAL6003)-plasminogen in 50 mM Tris buffer containing 20 mM NaCl, pH 8.0, 25°C. The fluorescence was monitored at an excitation wavelength of 298nm and an emission wavelength of 340nm in a FLUOROMAX fluorescence spectrophotometer (Jobin Yvon, Inc., Edison, NJ); after each addition of εACA, the solution was allowed to equilibrate until no further changes in fluorescence were observed.

The resulting fluorescence values were corrected for dilution and plotted versus the concentration of εACA over a range of 0 - 50 µM εACA. Data were fitted by non-linear regression to obtain a K_d of about 19 µM.

One property of plasminogen is its ability to bind fibrin. In order to determine whether (TAL6003)-plasminogen retains the ability to interact with fibrin, its fibrin-binding properties was tested in a microtiter plate assay in which binding of (TAL6003)-plasminogen to fibrin was assessed by its subsequent activation by tPA and resulting clot lysis. For this purpose, 100 µl of 5mg/ml fibrinogen was polymerized with thrombin in each well of a microtiter plate. Various concentrations of (TAL6003)-plasminogen was added on top of the fibrin clots and incubated for 1 hour at 37°C. The plate was washed extensively with PBS while the fibrin clots are still intact and attached to the wells. After washing, a 0.1 mg/ml solution of tPA was added to each well and the plate was incubated 2 hours at 37°C. As a result, some of the clots were completely dissolved and some were partially dissolved, while wells

with very low amounts of (TAL6003)-plasminogen and control wells remained practically intact. The degree of fibrinolysis was monitored by measuring the 280nm absorbance of remainders of the initial clots reconstituted in 1M NaOH. The absorbance values were plotted as a function of (TAL6003)-plasminogen concentration.

As shown in Figure 8, the binding of (TAL6003)-plasminogen to fibrin followed a classic, sigmoidal binding curve. Using this assay, it was found that (TAL6003)-plasminogen binds fibrin with affinity comparable to that of full-length plasminogen and the C_{50} of this interaction (~0.3 μM) is comparable to the K_d of fibrin-binding of full-length plasminogen.

These experiments indicate that (TAL6003)-plasminogen can bind fibrin. Further, at least the interaction of (TAL6003)-plasminogen with lysine-Sepharose, its ability to bind εACA with the expected Kd, its ability to bind fibrin, and its ability to be activated by a plasminogen activator all indicated that this molecule was produced in the *E. coli* system in a fully functional form.

(TAL6003)-plasmin Purification and Formulation

The addition of SK to the purified (TAL6003)-plasminogen solution effects the conversion of (TAL6003)-plasminogen to (TAL6003)-plasmin. The protein was concentrated to 2 mg/ml and diluted 1:1 with 50% glycerol to produce a 1mg/ml solution in 25% glycerol. The solution was brought to room temperature and streptokinase was added at a 1:100 molar ratio of SK: (TAL6003)-plasminogen. The reaction was incubated without stirring at room temp for 4.5 hrs. The reaction was then slowed down by addition of NaCl to a 0.5 M final concentration. Analysis of activation by SDS-PAGE indicated a 90% yield of activated protein.

Activated (TAL6003)-plasmin was purified by Benzamidine Affinity Chromatography. The purpose of benzamidine affinity purification was the separation of unactivated (TAL6003)-plasminogen and impurities, including (TAL6003)-plasmin degradation products, from active (TAL6003)-plasmin. The SK activation solution was applied to an equilibrated Benzamidine-SEPHAROSE 4 Fast Flow column. The (TAL6003)-plasmin, both clipped and intact, was captured by the affinity resin while the aforementioned impurities flowed through the column. The column was washed with the equilibration buffer until the absorbance at 280 nm

reached baseline. The bound (TAL6003)-plasmin was then eluted using a low pH εACA step to strip all remaining protein from the column. Typical yields were 75%, with protein that is 95% active as measured by chromogenic plasmin potency assay.

Because (TAL6003)-plasmin, similar to full-length plasmin, is prone to auto-degradation at physiological pH, pH 3.6 was chosen for the final formulation (acidified with acetic acid-saline). As shown previously for plasmin by Novokhatny *et al.*, J Thromb Haemost., 1(5):1034-41 (2003), incorporated by reference, and confirmed in experiments with (TAL6003)-plasmin, this low buffering-capacity, low pH formulation not only allows safe storage of active plasmins for prolonged periods of time, but is also compatible with parenteral administration of these direct thrombolytics. When mixed with plasma or neutral pH buffers, (TAL6003)-plasmin is quickly re-activated.

Enzymatic Properties of (TAL6003)-plasmin

The amidolytic activity of (TAL6003)-plasmin was examined using the plasmin substrate D-Val-Leu-Lys-p-nitroanilide (S-2251) (DiaPharma, West Chester, OH).

For (TAL6003)-plasmin, at pH 7.4, 25°C in PBS buffer, the Michaelis-Menten constant (K_M) for S-2251 was found to also be 141 μM (Table 3). The kcat for the preparation was found to be about 725 min^{-1}. Using 4-nitrophenyl 4-guanidinobenzoate hydrochloride (pNPGB) titration (Chase, T. and E. Shaw, *Methods Enzymol. 197*:20-27(1970)), the percent of functional active sites was found to be 67%. Correcting kcat for percent active sites, a kcat of about 725 min^{-1} was determined. This value was very close to the value determined in the same assay for full-length plasmin, 820 +/-23 min-1 and for micro-plasmin (lacking all five kringles), 795 +/- 24 min^{-1}. These data indicate that presence or absence of kringles does not affect the catalytic activity of the serine protease domain.

Table 3. Steady-state kinetic parameters for various plasmin species with substrate S-2251, in PBS buffer, pH 7.4, 25 °C.

	K_M	K_{CAT}
plasmin	220 +/- 9 μM	820 +/- 23 min^{-1}
mini-plasmin	160 +/- 30 μM	770 +/- 70 min^{-1}
micro-plasmin	145 +/- 13 μM	795 +/- 24 min^{-1}
(TAL6003)-plasmin	141 +/- 9 μM	725 min^{-1}

The rate of inhibition of (TAL6003)-plasmin by α_2-antiplasmin was determined to be $1.8 \pm 0.06 \times 10^7$ $M^{-1}s^{-1}$ using the method of Wiman and Collen (Wiman, B. and D. Collen, *Eur. J. Biochem. 84*:573-578 (1978)) in which plasmin and α_2-antiplasmin are mixed then assayed for S-2251 activity at specific time points (Table 4). This value is comparable to reported values for plasmin of 2.5×10^7 $M^{-1}s^{-1}$ (from Anonick, *et al.*, *Thrombosis Res. 59*:449 (1990)).

Table 4. Inhibition rates for various plasmin species and inhibitors were determined at 22°C in PBS buffer, pH 7.4.

	α_2-antiplasmin
plasmin	$2.5 +/- 0.5 \times 10^7$ $M^{-1}s^{-1}$ (lit.)
mini-plasmin	$2.4 +/- 0.5 \times 10^5$ $M^{-1}s^{-1}$
micro-plasmin	$1.8 +/- 0.2 \times 10^5$ $M^{-1}s^{-1}$
(TAL6003)-plasmin	$1.8 \pm 0.06 \times 10^7$ $M^{-1}s^{-1}$

The same experiments conducted with micro-plasmin revealed α_2-antiplasmin inhibition rates of 1.8×10^5 M^{-1} s^{-1} and 3.1×10^5 M^{-1} s^{-1} in two separate experiments. The rate of α_2-antiplasmin inhibition of mini-plasmin (mini-plasmin domain composition, K5-SP) was determined to be 2.4×10^5 M^{-1} s^{-1}. These data are in reasonable agreement with literature values for micro- and mini-plasmin and show that inhibition of (TAL6003)-plasmin by α_2-antiplasmin is 40-fold faster than the inhibition of either micro-plasmin or mini-plasmin. Thus, these results indicate that (TAL6003)-plasmin should be rapidly inhibited by α_2-antiplasmin due to the presence of kringle 1 in its structure. Overall, the data presented in this section show that the enzymatic and inhibitory properties of (TAL6003)-plasmin is similar to full-length plasmin.

Literature values are taken from Anonick, *et al.*, *Thrombosis Res. 59*:449(1990). All rates were measured according to the methods published in Anonick, *et al.*

***In Vitro* Fibrinolytic Efficacy**

The fibrinolytic efficacy of (TAL6003)-plasmin was tested in an *in vitro* model of clot lysis assay using the following experimental protocol.

In vitro comparison of the thrombolytic efficacy of (TAL6003)-plasmin with plasma-derived plasmin. Equimolar amounts of plasma-derived plasmin (0.25 mg/ml) and (TAL6003)-plasmin (0.11 mg/ml) were mixed with blood clots in the test

tube and degree of clot lysis was monitored by A_{280} absorbance of material released from the clot.

The concentrations of plasmin or (TAL6003)-plasmin required to overcome plasma inhibitors in the presence of fibrin and initiate clot lysis are shown in Figure 9.

What is claimed is:

1. A polynucleotide comprising a nucleotide sequence encoding a polypeptide having

a) a single N-terminal kringle domain homologous to a kringle domain of native human plasminogen, wherein the last four amino acid residues within the kringle domain are V,P,Q, and C; and

b) a C-terminal domain activation site and serine protease domain homologous to the corresponding domains in human plasminogen; wherein the polypeptide binds to immobilized lysine.

2. The polynucleotide of claim 1, wherein the single N-terminal kringle domain is homologous to kringle 1 or kringle 4 of native human plasminogen.

3. The polynucleotide of claim 1, wherein the encoded polypeptide is at least 90% or 95%, or 98% identical to the sequence shown in SEQ ID NO: 2.

4. The polynucleotide of claim 1, wherein the encoded polypeptide is at least 98% identical to the sequence shown in SEQ ID NO: 2.

5. The polynucleotide of claim 1, wherein the encoded polypeptide is the sequence shown in SEQ ID NO: 2.

6. The polynucleotide of claim 1, wherein the nucleotide sequence of the polynucleotide is the sequence shown in SEQ ID NO:1, or a degenerate variant thereof.

7. The polynucleotide of claim 1, wherein the single N-terminal kringle domain is at least 90% identical to the kringle 1 or kringle 4 domain of native human plasminogen; and the C-terminal domain is at least 90% identical to the activation site and serine protease domain of human plasminogen.

8. The polynucleotide of claim 1, wherein the immobilized lysine is lysine bound to a solid support matrix selected from the group consisting of lysine-agarose, lysine-hydrogel, lysine-cross-linked agarose.

9. The polynucleotide of claim 8, wherein the immobilized lysine is lysine-cross-linked agarose.

10. The polynucleotide of claim 1, wherein the polypeptide exhibits a lower binding affinity for fibrinogen than the binding affinity for fibrinogen of mini-plasmin.

11. The polynucleotide of claim 1, wherein the polypeptide exhibits higher binding affinity for partially cleaved fibrin than the binding affinity for partially cleaved fibrin of mini-plasmin.

12. The polynucleotide of Claim 1, wherein the polypeptide has reduced immunogenicity as compared to a reference polypeptide, wherein the reference polypeptide has a primary amino acid sequence identical to the primary amino acid sequence of the polypeptide with the proviso that the last four amino acid residues of the single N-terminal kringle domain of the reference polypeptide is not V,P,Q, and C.

13. A polypeptide comprising:

a) a single N-terminal kringle domain homologous to a kringle domain of native human plasminogen, wherein the last four amino acid residues within the kringle domain are V,P,Q, and C; and

b) a C-terminal domain activation site and serine protease domain homologous to the corresponding domains in human plasminogen; wherein the polypeptide binds to immobilized lysine.

14. The polypeptide of claim 13, wherein the single N-terminal kringle domain is homologous to kringle 1 or kringle 4 of native human plasminogen.

15. The polypeptide of claim 13, wherein the polypeptide exhibits a fibrinolytic activity that is inhibited by α_2-antiplasmin at a rate of inhibition that is at least about 5-fold faster than the rate of inhibition of the fibrinolytic activity of mini-plasmin by α_2-antiplasmin.

16. The polypeptide of claim 15, wherein the rate of inhibition is at least about 10-fold, 20-fold, 30-fold, or 40-fold faster than the rate of inhibition of mini-plasmin.

17. The polypeptide of Claim 13, wherein the polypeptide is at least 90% or 95%, or 98% identical to the sequence shown in SEQ ID NO: 2.

18. The polypeptide of Claim 13, wherein the single N-terminal kringle domain is at least 90% identical to the kringle 1 or kringle 4 domain of native human plasminogen; and the C-terminal domain is at least 90% identical to the activation site and serine protease domain of human plasminogen.

19. The polypeptide of Claim 13, wherein the single N-terminal kringle domain has at least one residue greater amino acid sequence identity with kringle 1 or kringle 4 of native human plasminogen than with kringle 5 of native human plasminogen, and wherein conservative substitutions of the single N-terminal kringle domain relative to the native sequences of kringles 1 and 4 of human plasminogen are not considered as differing from the native sequences for purposes of the identity comparison with kringle 5.

20. The polypeptide of Claim 13, wherein the polypeptide has an amino acid sequence as shown in SEQ ID NO: 2, and conservative substitutions thereof.

21. The polypeptide of Claim 13, wherein the polypeptide has an arginine residue at a relative position analogous to that of position 85 of the amino acid sequence shown in SEQ ID NO: 2.

22. The polypeptide of Claim 13, wherein the polypeptide has reduced immunogenicity as compared to a reference polypeptide, wherein the reference polypeptide has a primary amino acid sequence identical to the primary amino acid sequence of the polypeptide with the proviso that the last four amino acid residues of the singl

23. An expression vector comprising the polynucleotide of claim 1.

24. A cultured cell comprising the expression vector of claim 23.

e N-terminal kringle domain of the reference polypeptide is not V,P,Q, and C.

25. A method for making one or more recombinant plasmin polypeptides having different N-termini, the method comprising:

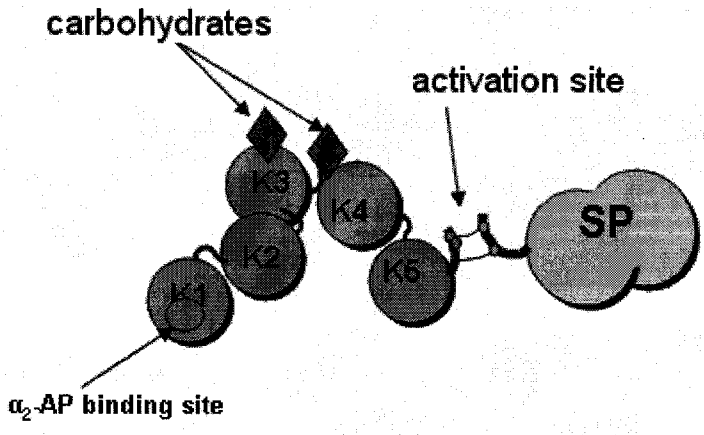
a) providing a polypeptide having a single N-terminal kringle domain homologous to a kringle domain of native human plasminogen, wherein the last four amino acid residues within the kringle domain are V,P,Q, and C; and a C-terminal domain activation site and serine protease domain homologous to the corresponding domains in human plasminogen; wherein the polypeptide binds to immobilized lysine.

b) contacting the polypeptide provided in step a) with a protease under conditions sufficient to cleave one or more peptide bonds thereby forming one or more recombinant plasmin polypeptides having different N-termini.

26. The method of Claim 25, wherein the polypeptide has an amino acid sequence shown in SEQ ID NO: 2.

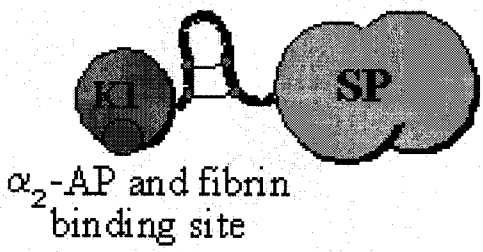
27. The method of Claim 25, wherein providing comprises expressing a DNA sequence shown in SEQ ID NO:1, or a degenerate variant thereof, in *E. Coli*.

FIG. 1



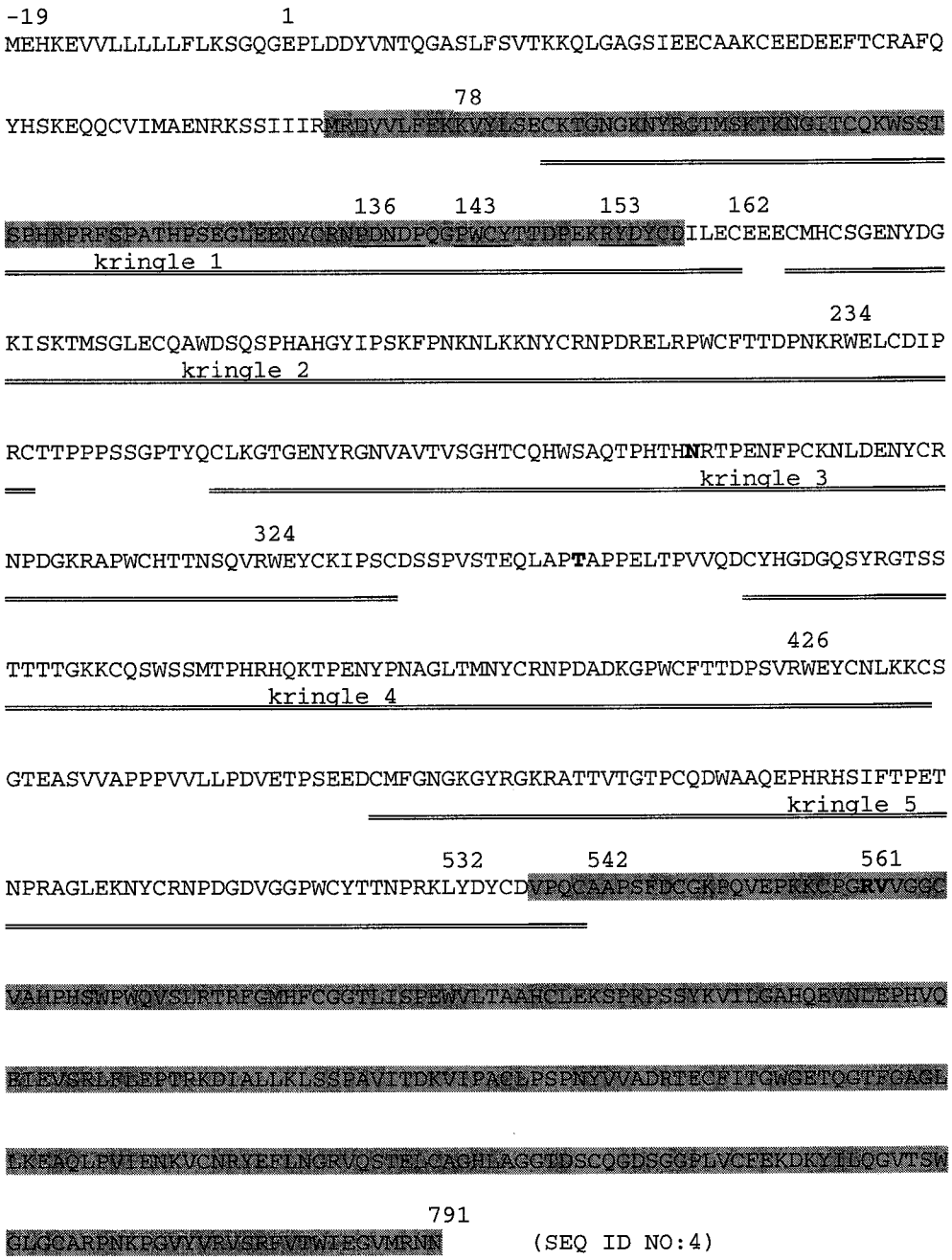
Plasmin

FIG. 2



(TAL6003)-plasminogen

FIG. 3


-19
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136
143
153
162
SPHRPRFSPATHPSEGLEENYCRNPDNDPQGPWCYTTDPEKRYDYCDILECEEECMHCSGENYDG
kringle 1
234
KISKTMSGLECQAWDSQSPHAHGYIPSKFPNKNLKKNYCRNPDRELRPWCFTTDPNKRWELCDIP
kringle 2
RCTTPPPSSGPTYQCLKGTGENYRGNVAVTVSGHTCQHWSAQTPHTHNRTPENFPCKNLDENYCR
kringle 3
324
NPDGKRAPWCHTTNSQVRWEYCKIPSCDSSPVSTEQLAPTAPPELTPVVQDCYHGDGQSYRGTSS
426
TTTTGKKCQSWSSMTPHRHQKTPENYPNAGLTMNYCRNPDADKGPWCFTTDPSVRWEYCNLKKCS
kringle 4
GTEASVVAPPPVVLLPDVETPSEEDCMFGNGKGYRGKRATTVTGTPCQDWAAQEPHRHSIFTPET
kringle 5
532
542
561
NPRAGLEKNYCRNPDGDVGGPWCYTTNPRKLYDYCDVPQCAAPSFDCGKPQVEPKKCPGRVVGGC
VAHPHSWPWQVSLRTRFGMHFCGGTLISPEWVLTAAHCLEKSPRPSSYKVILGAHQEVNLEPHVQ
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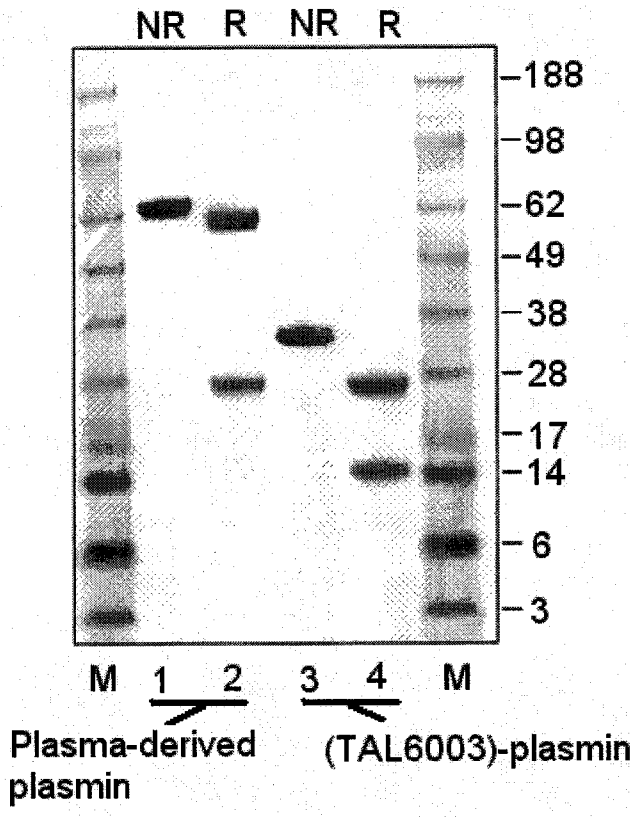
LKEAQLPVIENKVCNRYEFLNGRVQSTELCAGHLAGGTDSCQGDSGGPLVCFEKDKYILQGVTSW
791
GLGCARPNKPGVYVRVSRFVTWIEGVMRNN
(SEQ ID NO:4)

FIG. 4

```
HK1  CKTGNGKNYR GTMSKTKNGI TCQKWSSTSP HR-PRFSPAT HPSEGLEENY
HK2  CMHCSGENYD GKISKTMSGL ECQAWDSQSP HA-HGYIPSK FPNKNLKKNY
HK3  CLKGTGENYR GNVAVTVSGH TCQHWSAQTP HT-HNRTPEN FPCKNLDENY
HK4  CYHGDGQSYR GTSSTTTTGK KCQSWSSMTP HR-HQKTPEN YPNAGLTMNY
HK5  CMFGNGKGYR GKRATTVTGT PCQDWAAQEP HRHSIFTPET NPRAGLEKNY

(con't)
HK1  CRNPDNDPQG PWCYTTDPEK RYDYCDILEC (SEQ ID NO:11)
HK2  CRNPDRE-LR PWCFTTDPNK RWELCDIPRC (SEQ ID NO:12)
HK3  CRNPDGK-RA PWCHTTNSQV RWEYCKIPSC (SEQ ID NO:13)
HK4  CRNPDAD-KG PWCFTTDPSV RWEYCNLKKC (SEQ ID NO:14)
HK5  CRNPDGDVGG PWCYTTNPRK LYDYCDVPQC (SEQ ID NO:15)
```

FIG. 5


NR R NR R
188
98
62
49
38
28
17
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6
3
M 1 2 3 4 M
Plasma-derived plasmin
(TAL6003)-plasmin

FIG. 6

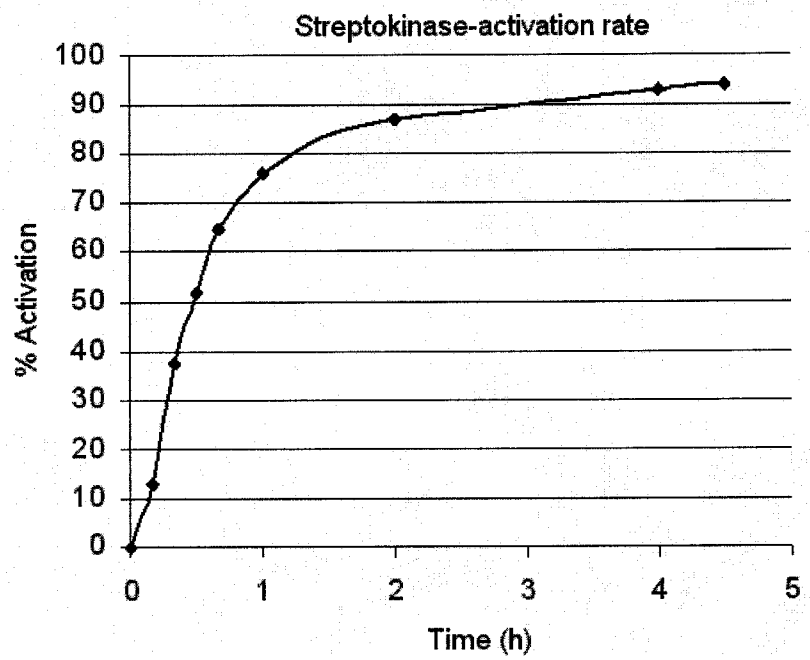

Streptokinase-activation rate
% Activation
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
Time (h)

FIG. 7

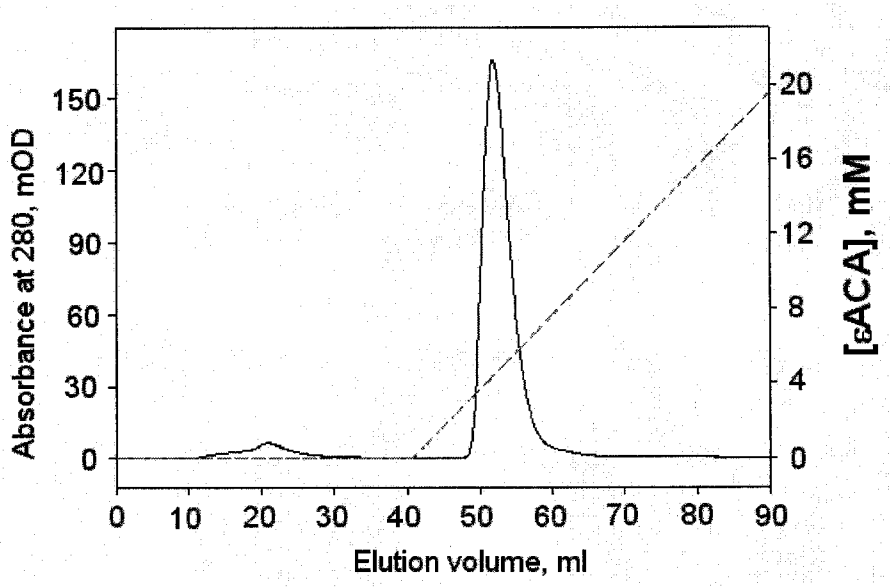

Absorbance at 280, mOD
150
120
90
60
30
0
[εACA], mM
20
16
12
8
4
0
0
10
20
30
40
50
60
70
80
90
Elution volume, ml

FIG. 8

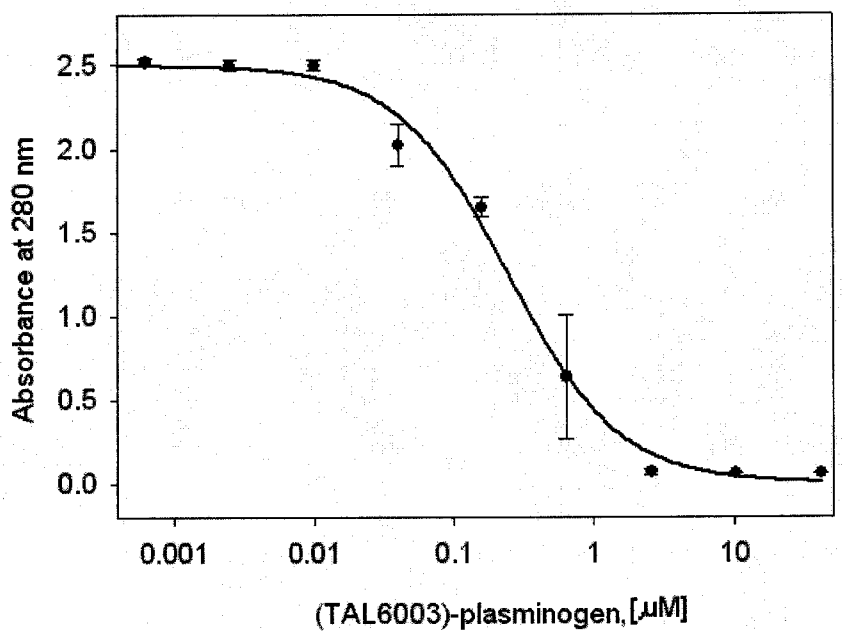

Absorbance at 280 nm
2.5
2.0
1.5
1.0
0.5
0.0
0.001
0.01
0.1
1
10
(TAL6003)-plasminogen,[μM]

FIG. 9

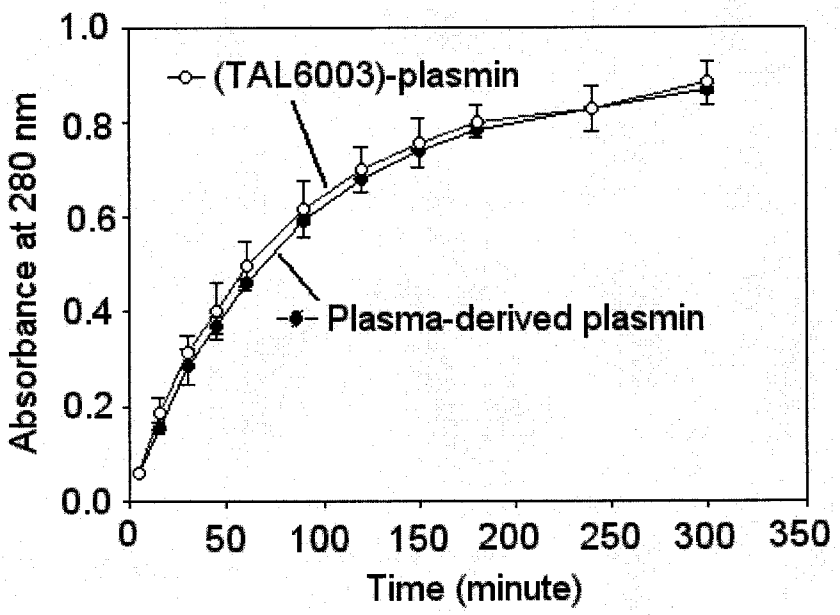
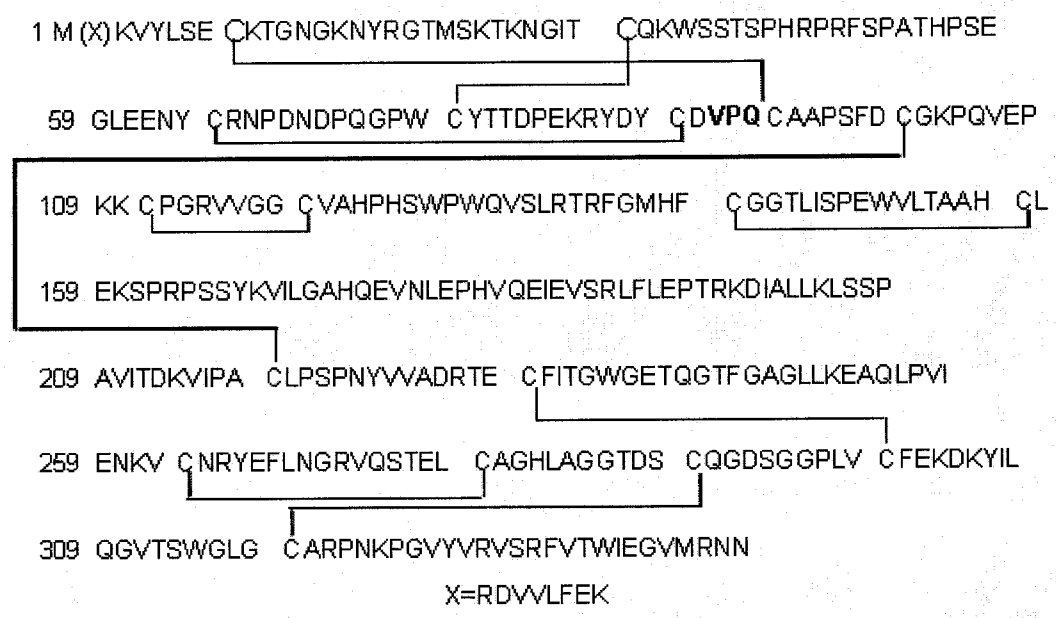

Absorbance at 280 nm
1.0
0.8
0.6
0.4
0.2
0.0
(TAL6003)-plasmin
Plasma-derived plasmin
0
50
100
150
200
250
300
350
Time (minute)

FIGURE 10



(SEQ ID NO:2)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2008/084645

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/68 C12N15/09

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K C12N

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, Sequence Search, WPI Data, EMBASE, MEDLINE, BIOSIS, FSTA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2007/047874 A (TALECRIS BIOTHERAPEUTICS INC [US]; SCUDERI JR PHILIP [US]; ARORA VIKRA) 26 April 2007 (2007-04-26) page 28, line 1 - page 34, line 11 figures 1,2 sequences 1,2	1-27
X	WO 2005/105990 A (TALECRIS BIOTHERAPEUTICS INC [US]; HUNT JENNIFER AUDREY [US]; NOVOKHAT) 10 November 2005 (2005-11-10) page 22, line 1 - page 28, line 6; sequences 1,2	1-27
X	WO 2004/052228 A (THROMB X NV [BE]; PAKOLA STEVE [US]; DE SMET MARC [NL]) 24 June 2004 (2004-06-24) figure 3; sequences 7,8 example 3	1,3,8,9, 13,17, 18,22-25

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
9 March 2009	17/03/2009

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Voigt-Ritzer, Heike

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2008/084645

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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X	WO 02/50290 A (THROMB X NV [BE]; COLLEN DESIRE JOSE [GB]; NAGAI NUBUO [BE]; LAROCHE Y) 27 June 2002 (2002-06-27) examples 1,3; sequences 5,6	1,8,9, 13,22-25
X	MEDYNSKI DAN ET AL: "Refolding, purification, and activation of miniplasminogen and microplasminogen isolated from E. coli inclusion bodies" PROTEIN EXPRESSION AND PURIFICATION, ACADEMIC PRESS, SAN DIEGO, CA, vol. 52, no. 2, 26 October 2006 (2006-10-26), pages 395-402, XP002490137 ISSN: 1046-5928 the whole document	1,8,9, 13,22-25
A	LIN L-F ET AL: "Epsilon amino caproic acid inhibits streptokinase-plasminogen activator complex formation and substrate binding through kringle-dependent mechanisms" BIOCHEMISTRY, AMERICAN CHEMICAL SOCIETY, EASTON, PA.; US, vol. 39, no. 16, 25 April 2000 (2000-04-25), pages 4740-4745, XP002348009 ISSN: 0006-2960 page 4740, column 2, line 6 - line 7	1-27

INTERNATIONAL SEARCH REPORT

Information on patent family members

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