

Description

The present invention is directed to particular variants of plasminogen activators, to methods for preparing such, and to methods and compositions utilizing such variants for producing pharmaceutically active compositions with unexpectedly improved therapeutic and physicochemical characteristics, particularly improved circulating half-life and clearance rates.

Plasminogen activators are enzymes that activate the zymogen plasminogen to generate the serine proteinase plasmin (by cleavage at Arg561-Val562) that degrades various proteins, including fibrin. Among the plasminogen activators studied are streptokinase, a bacterial protein, urokinase, an enzyme synthesized in the kidney and elsewhere and originally extracted from urine, and human tissue plasminogen activator (t-PA), an enzyme produced by the cells lining blood vessel walls.

The mechanism of action of each of these plasminogen activators differs: Streptokinase forms a complex with plasminogen, generating plasmin activity, urokinase cleaves plasminogen directly, and t-PA forms a ternary complex with fibrin and plasminogen, leading to plasminogen activation in the locality of the clot.

t-PA has been identified and described as a particularly important and potent new biological pharmaceutical agent that has shown extraordinary results in the treatment of vascular diseases, such as myocardial infarction, due to its high fibrin specificity and potent ability to dissolve blood clots *in vivo*.

t-PA has been the subject of numerous scientific and patent application disclosures. Although its existence prompted numerous investigations by several scientific groups, it was first identified as a substantially pure isolate from a natural source, and tested for requisite plasminogen activator activity *in vivo*, by Collen et al., European Patent Application Publ. No. 41,766, published 16 December 1981. See also the corresponding scientific publication, Rijken et al., *J. Biol. Chem.*, 256: 7035 (1981).

Subsequently, t-PA was fully identified and characterized by underlying DNA sequence and deduced amino acid sequence based on successful work employing recombinant DNA technology resulting in large quantities of t-PA in a distinct milieu. This work was reported by Pennica et al., *Nature*, 301: 214 (1983)) and in European Patent Application Publ. No. 93,619, published 9 November 1983.

Using the latter disclosure as a basic tool, numerous other researchers have reported on the thus enabled preparation of the molecule via recombinant DNA technology. Certain of these researchers also have disclosed publicly the potential of variants of the basic structure, mentally foreseeing derivatives that may vary in overall biological or pharmacokinetic effects. The resultant public disclosures for the most part have been prophetic and equivocal in terms of actual overall biological or pharmacological results.

Analogous endeavors in the laboratories that succeeded first in producing t-PA recombinantly have been recorded factually in terms of confirmed molecular characterization and observed biological effect, both in the scientific literature and in various patent applications, notably those cited. In all events, the trend seems to favor research attempting to modify the basic structure of t-PA to explore and exploit fully its commercial potential according to various biologically based endpoints.

Based partly upon such research and disclosures, it seems now clear that the t-PA molecule contains five domains (stretches of amino acid sequence) that have been defined with reference to homologous or otherwise similar structures identified in various other proteins such as trypsin, chymotrypsin, plasminogen, prothrombin, fibronectin, and epidermal growth factor (EGF). These domains have been designated, starting at the N-terminus of the amino acid sequence of t-PA, as 1) the finger region (F) that has variously been defined as including amino acids 1 to about 44, 2) the growth factor region (G) that has been variously defined as stretching from about amino acids 45 to 91 (based upon its homology with EGF), 3) kringle one (K1) that has been defined as stretching from about amino acid 92 to about amino acid 173, 4) kringle two (K2) that has been defined as stretching from about amino acid 180 to about amino acid 261, and 5) the so-called serine protease domain (P) that generally has been defined as stretching from about amino acid 264 to the C-terminal end of the molecule. These domains are situated generally adjacent to one another, or are separated by short "linker" regions, and account for the entire amino acid sequence of from 1 to 527 amino acids of the putative mature form of t-PA.

Each domain has been described variously as contributing certain specific activity. The finger domain has been variously described as containing a sequence of at least major importance for high binding affinity to fibrin. (This activity is thought important for the high specificity that t-PA displays with respect to clot lysis at the locus of a fibrin-rich thrombus.) The growth factor-like region likewise has been associated with cell surface binding activity, at least with respect to urokinase. The kringle 2 region has also been strongly associated with fibrin binding and with the ability of fibrin to stimulate the activity of t-PA. The serine protease domain is responsible for the plasminogen activating activity of t-PA.

Potential N-linked glycosylation sites exist in the molecule at amino acid positions 117, 184, 218, and 448, numbered in accordance with native, mature t-PA. The site at amino acid 218 is not glycosylated in native t-PA. The glycosylation site at amino acid 117 has been characterized as being a high mannose type, while the other two sites display so-called complex oligosaccharide structures. Sites 117 and 448 seem always to be glycosylated, when the molecule is derived from a host cell capable of effecting glycosylation, while site 184 is thought to be glycosylated in about 50 percent of the molecules.

The glycosylated/unglycosylated phenomenon at site 184 has been demonstrated via SDS-PAGE analysis, where two bands can be seen, one associated with glycosylated molecules at position 184, and the other unglycosylated molecules at position 184. These bands have been designated as Type I and Type II t-PA, respectively. This partial glycosylation pattern may be the result of site 184 being situated in a conformationally sheltered position in the protein.

Another locus of scientific note is the so-called proteolytic cleavage site within the region defined by amino acids 275 to about 279, and more particularly, the bond between amino acid 275 and 276 of the native molecule. Mutagenesis at this site so as to make it less susceptible to proteolytic degradation creates a molecule that remains in a single-, or one-chain, form that is thought to have certain advantages biologically and commercially.

As mentioned above, another plasminogen activator, urokinase, has been purified from human urine and human kidney cell culture fluids (Gunzler et al., *Hoppe-Seyler's Z. Physiol. Chem.*, 363: 1155-1165 (1982) and Steffens, et al., *Hoppe Seyler's Z. Physiol. Chem.*, 363: 1043-1058 (1982)) and produced recombinantly (EPO Publ. No. 154,272 and Holmes et al., *Biotechnology*, 3: 923-929 (1985)).

Urokinase contains 411 amino acids and is produced with an N-terminal leader sequence that is cleaved during maturation, resulting in the production of prourokinase. Prourokinase is in turn cleaved by plasmin to yield two urokinase species: one of molecular weight 54,000 daltons and one of molecular weight 33,000 daltons.

Urokinase has three identifiable domains: a growth factor domain encompassing positions 5 to 49, a kringle domain embracing positions 50 to 136, and a serine protease domain encompassing positions 159 to 411. Prourokinase similarly consists of these three domains. See Gunzler et al., *supra*. The enzymatically active amino acid residues in urokinase have been located at positions 204, 255, and 356, and an N-linked glycosylation site occurs at Asn302.

When used in large doses, urokinase results in degradation and activation of coagulation and fibrinolysis factors that leads to bleeding. In contrast, the precursor form of human urokinase, prourokinase, described in EPO Publ. No. 139,447 and in *J. Biol. Chem.*, 260: 12377 (1985), dissolves thrombi without inducing any substantial bleeding. *Cell Struc. Func.*, 10: 151 (1985).

A review article on plasminogen activators and second-generation derivatives thereof is Harris, *Protein Engineering*, 1: 449-458 (1987).

Natural t-PA has a plasma half-life of typically about six minutes or less, when administered to patients in therapeutically effective amounts. Prourokinase has a similar half-life. Such a half-life is desirable under certain situations, for example, when acute aggressive therapy of a life-threatening disease such as myocardial infarction or pulmonary embolism is undertaken. In this high-risk situation, patients may be treated who have significant or unrecognized potential for uncontrolled bleeding. If such bleeding occurred, drug administration could be stopped and the causative t-PA levels would be rapidly depleted by high clearance rates.

However, in other circumstances, for example, in the treatment of myocardial infarction following reperfusion, the desired therapeutic regimen is less aggressive and of extended duration (4 to 12 hours). A long half-life form of t-PA can be perceived as a more desirable, efficient and convenient treatment in patients who are not in life-threatening situations. Moreover, a t-PA of longer half-life would be desirable as an agent for bolus administration. For example, because ambulance technicians generally do not have infusion capability available, it would be much more desirable to employ t-PA-like agents having greater half-lives.

All of the defined domains and glycosylation sites, and the one-chain/two-chain cleavage site of t-PA have been described as having specific potential biological activity components. For example, removal of a substantial portion or all of the finger domain results in a molecule with substantially diminished fibrin binding characteristics, albeit in return there is a decrease in the overall rate of clearance of the resultant entity.

Modification of the native molecule so as to destroy the one-chain to two-chain cleavage site, as such, results in a molecule with somewhat altered biological activity and more stability while the fibrin binding and fibrin stimulation are increased relative to two-chain t-PA.

Glycosylation mutants at 117-119, 184-186, and 448-450 exhibited higher specific activity as the mole percent carbohydrate was reduced. See EPO Pub. No. 227,462. Further, the t-PA mutants with Asn119, Ala186 and Asn450, which have the N-glycosylation sites selectively removed by DNA modification but contain residual O-linked carbohydrate, were found to be about two-fold as potent as melanoma t-PA in an *in vitro* lysis assay. See EPO Publ. No. 225,286.

However, alteration of the glycosylation sites, and in particular that at amino acid 117, seems invariably to result in a molecule having affected solubility characteristics that may result additionally in an altered circulating half-life pattern and/or fibrin binding characteristics.

When the growth factor domain of t-PA is deleted, the resultant mutant is still active and binds to fibrin, as reported by A. J. van Zonneveld et al., *Thrombos. Haemostas.*, 54 (1) 4 (1985). Various deletions in the growth factor domain have also been reported in the patent literature. See EPO Publ. No. 241,209 (des-51-87), EPO Publ. No. 241,208 (des-51-87 and des-51-173), PCT 87/04722 (deletion of all or part of the N-terminal 1-91), EPO Publ. No. 231,624 (all of growth factor domain deleted), and EPO Publ. No. 242,836 and Jap. Pat. Appl. Kokai No. 62-269688 (some or all of the growth factor domain deleted).

It has further been shown that t-PA can be modified both in the region of the first kringle domain and in the growth factor domain, resulting in increased circulatory half-life. See EPO Pat. Publ. No. 241,208 published October 14, 1987. The region between amino acids 51 and 87, inclusive, can be deleted from t-PA to result in a variant having slower clearance from plasma. Browne et al., *J. Biol. Chem.*, 263: 1599-1602 (1988). Also, t-PA can be modified, without adverse biological effects, in the region of amino acids 67 to 69 of the mature, native t-PA, by deletion of certain amino acid residues or replacement of one or more amino acids with different amino acids. See EPO Pat. Publ. No. 240,334 published October 7, 1987. Moreover, when the entire or a partial epidermal growth factor domain of the human prourokinase protein is deleted or replaced by one or more different amino acid residues, the resultant variants exhibit increased half-life in blood. See EPO Pat. Publ. No. 253,241 published January 20, 1988.

There is a current and continuing need in the art to identify specific sites within plasminogen activator molecules that can be modified to impart to the molecules improved pharmacokinetic characteristics over the native molecule. Such variant molecules would provide medical science important new alternatives in the treatment of cardiovascular disease and numerous other medical conditions that arise from thromboembolic occlusion of blood vessels.

Accordingly, it is an object of this invention to provide plasminogen activator molecules to patients requiring clot-dissolving agents that exhibit improved therapeutic and pharmaceutical characteristics.

Another object is to provide plasminogen activator molecules with a longer half-life and slower clearance rate from plasma relative to that of currently available clot-dissolving agents.

It is another object to provide for the treatment of conditions that admit the use of clot-dissolving agents having longer circulatory half-lives and longer clearance rates from plasma relative to natural t-PA, for example, conditions such as deep vein thrombosis or peripheral arterial thrombosis (peripheral vascular disease).

The present invention provides a fibrinolytically active human tissue plasminogen activator (t-PA) amino acid sequence variant that has N-linked or O-linked glycosylation at a glycosylation site which is not present in native human t-PA, said plasminogen activator variant exhibiting increased half-life and decreased clearance rate relative to native human t-PA.

In one preferred embodiment, such glycosylation is at a site containing a Asn-X-Ser or Asn-X-Thr tripeptidyl sequence of the variant, wherein X is any amino acid except proline.

In another embodiment, the plasminogen activator is glycosylated within its growth factor domain.

In still another embodiment, the plasminogen activator is t-PA, and the tyrosine residue at position 67, numbered in accordance with the native amino acid sequence of t-PA, is substituted with another amino acid capable of glycosylation, such as asparagine, serine, or threonine, preferably asparagine.

In other embodiments, this invention relates to a DNA sequence encoding the variant described above, replicable expression vectors capable of expressing the DNA sequence in a transformant host cell, and microorganisms and cell cultures transformed with the vector.

In yet another embodiment, the invention is directed to a composition for treating a vascular disease or condition comprising a therapeutically effective amount of the variant herein in admixture with a pharmaceutically acceptable carrier.

In still another embodiment, the invention provides a method of treating a vascular disease or condition in a patient comprising administering the above composition containing the variant to the patient.

The present invention is based, *inter alia*, upon specific successful research demonstrating that glycosylation at a site of the plasminogen activator that is not ordinarily glycosylated in the native molecule results in variants that have an extended circulatory half-life and slower clearance rate as compared with the

native material. The results are molecules that differ substantially from the native material in overall amino acid sequence, but exhibit pharmacokinetic properties to a degree permitting their commercialization in conjunction with or as an alternative to the native material.

Figure 1 depicts the N-linked glycosylation patterns of proteins produced from mammalian cells and yeast, where SA is sialic acid, gal is galactose, man is mannose, GlcNAc is N-acetyl glucosamine, and Asn is asparagine.

Figure 2 depicts the primary structure of t-PA showing the location of the five domains, the disulfide bridging, and the glycosylation sites.

Figure 3 depicts the primary structure of human urokinase showing the location of the three domains, the disulfide bridging, and the glycosylation site.

Figures 4a and 4b and 5a and 5b are schematic representations of a suitable method for the preparation of pCIS-t-PA, together with a description of certain of its prominent restriction sites.

Figure 6 is a schematic representation of a suitable method for the preparation of p7-1H, together with a description of certain of its prominent restriction sites.

Figure 7 shows *in vitro* clot lysis results and S-2251 results, expressed as a percent of native specific activity, for rt-PA and glycosylated N67 t-PA. Results show averages of several independent observations (clot lysis, four determinations; S-2251, two determinations). All were from material expressed transiently in 293 cells and quantified by ELISA.

Figure 8 shows fibrin binding of rt-PA and glycosylated N67 t-PA at a t-PA concentration of 10 ng/ml. Both were expressed transiently in 293 cells.

Figures 9A-9C show the reverse phase HPLC profiles of tryptic mapping analysis of reduced and carboxymethylated rt-PA, N67 tPA, and N-glycanase-treated (de-glycosylated) N67 tPA, respectively. All were expressed transiently in 293 cells.

Figure 10 shows the pharmacokinetic profiles, in rabbits, of rt-PA, des 1-44 t-PA, des 1-44E275 t-PA, des 1-44E275D184, and glycosylated N67 t-PA, all except N67 t-PA expressed in stable CHO cell lines. The N67 material was obtained via transient expression in 293 cells.

Detailed Description of the Preferred Embodiments

A. Definitions

As used herein, "plasminogen activator" refers to a protein that is capable of converting plasminogen to plasmin. Examples of such plasminogen activators include tissue-type plasminogen activator, urokinase, prourokinase, from any species, preferably human; streptokinase; and the like. These plasminogen activators may be produced, e.g., by recombinant cell culture systems, in bioactive forms. It will be understood that natural allelic variations exist and occur from individual to individual, demonstrated by (an) amino acid difference(s) in the overall sequence.

The expression "glycosylated at one or more regions that are not glycosylated in the corresponding native plasminogen activator" refers to glycosylation, whether N- or O-linked, whether from yeast or mammalian hosts, and whether of complex, high mannose, or hybrid structure, that occurs in regions not actually or potentially glycosylated in the native molecule. Such actual or potential positions are indicated by circles in Figures 1 and 2 for t-PA and urokinase, respectively. Thus, for example, while there are four potential sites for N-glycosidic linkage in the t-PA molecule (at Asn117, Asn184, Asn218, and Asn448), only three are actually glycosylated in the native t-PA (117, 184 (in 50% of the molecules) and 448). While potentially a glycosylation site, the Asn218-Pro-Ser site in t-PA is not utilized for carbohydrate attachment in mammalian cells because of the presence of the proline in the sequence. See G. Pohl et al., *Biochemistry*, **23**: 3701 (1984). In urokinase the glycosylation site occurs at position 302.

As used herein, "growth factor domain" refers to that region of the plasminogen activator that is structurally homologous with human and/or murine epidermal growth factors. See, e.g., Banyai et al., *FEBS Lett.*, **163**: 37 (1983). In t-PA, this region is from amino acids about 44 to about 91; in prourokinase, this region is from about the N-terminal serine to about the 49th amino acid residue threonine; and in urokinase, this region is from about 5 to about 49. The DNA encoding a major portion of this domain in t-PA (residues 51 to 86) and partially encoding residues 50 and 87 is contained on a single exon, as reported by Ny et al., *Proc. Natl. Acad. Sci. (U.S.A.)*, **81**: 5355 (1984).

As used herein, the terms "human tissue plasminogen activator," "human t-PA," and "t-PA" denote human extrinsic (tissue-type) plasminogen activator having two functional regions consisting of a protease domain that is capable of converting plasminogen to plasmin and a kringle-containing domain believed to be responsible for fibrin binding. These three terms therefore include polypeptides containing these

functional domains as part of the overall sequence.

A "two-chain cleavage site" in t-PA comprises at least the arginine residue at position 275. However, various amino acids adjacent to or within several residues of position 275 are also believed to be a part of the domain recognized by enzymes that convert plasminogen activator to its two-chain form. Thus, replacement of amino acids at positions other than 275 within the domain could result in mutant plasminogen activators that are resistant to conversion to the two-chain form.

In the particular embodiment, "single-chain plasminogen activator mutant" is a plasminogen activator that is resistant to conversion to the two-chain form. It is characterized by single or multiple amino acid substitutions at the two-chain activation site. As modified, such activation site is not enzymatically recognized, and, therefore, not hydrolyzed by enzymes that normally convert plasminogen activator to its two-chain form. A noted example of such a mutant is a molecule resistant to cleavage at the 275/276 cleavage site by imposed modifications in the 275 to 279 region, for example, having an amino acid other than arginine such as glycine or glutamic acid at position 275 and glutamic acid at position 275 and isoleucine at position 277 (designated G275, E275, and E275I277, respectively). These single-chain mutants are more fully described in European Patent Appl. 199574, 29 October 1986.

B. General Methods

1. Glycosylation

The plasminogen activator amino acid sequence variant must contain at least one amino acid sequence that can be glycosylated through an O- or N-linkage and that is not normally glycosylated in the native molecule.

If N-linked glycosylation is contemplated, the glycosylation site in the variant is a tripeptidyl sequence of the formula: asparagine-X-serine or asparagine-X-threonine, wherein asparagine is the acceptor and X is any of the twenty genetically encoded amino acids except proline, which prevents glycosylation. See D.K. Struck and W.J. Lennarz, in The Biochemistry of Glycoproteins and Proteoglycans, ed. W.J. Lennarz, Plenum Press, 1980, p. 35; R.D. Marshall, Biochem. Soc. Symp., 40: 17 (1974), and Winzler, R.J., in Hormonal Proteins and Peptides (ed. Li, C.I.) p. 1-15 (Academic Press, New York, 1973). The amino acid sequence variant herein is modified by either inserting the appropriate amino acid(s) at the proper site(s) to effect the glycosylation or substituting for the amino acid(s) at the appropriate site(s) the appropriate amino acids to effect glycosylation.

If O-linked glycosylation is to be employed, O-glycosidic linkage occurs in animal cells between N-acetylgalactosamine, galactose, or xylose and one of several hydroxyamino acids, most commonly serine or threonine, but also in some cases a 5-hydroxyproline or 5-hydroxylysine residue placed in the appropriate region of the molecule.

Glycosylation patterns for proteins produced by mammals are described in detail in The Plasma Proteins: Structure, Function and Genetic Control, F.W. Putnam, ed., 2nd edition, volume 4 (Academic Press, New York, 1984), p. 271-315. In this chapter, asparagine-linked oligosaccharides are discussed, including their subdivision into at least three groups referred to as complex, high mannose, and hybrid structures, as well as O-glucosidically linked oligosaccharides.

Chemical and/or enzymatic coupling of glycosides to proteins can be accomplished using a variety of activated groups, for example, as described by Aplin and Wriston in CRC Crit. Rev. Biochem., pp. 259-306 (1981). The advantages of the chemical coupling techniques are that they are relatively simple and do not need the complicated enzymatic machinery required for natural O- and N-linked glycosylation. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine or histidine, (b) free carboxyl groups such as those of glutamic acid or aspartic acid, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described more fully in PCT WO 87/05330 published September 11, 1987.

Glycosylation patterns for proteins produced by yeast are described in detail by Tanner and Lehle, Biochim. Biophys. Acta, 906(1): 81-99 (1987) and by Kukuruzinska et al., Annu. Rev. Biochem., 56: 915-944 (1987).

In addition, Figure 1 depicts a comparison of the N-linked glycosylation patterns of proteins produced in mammalian cells versus yeast.

2. Amino Acid Sequence Variants

For purposes of discussing the variants herein, reference is made to Figures 2 and 3, which respectively illustrate the primary structures of t-PA and human urokinase.

5 In Figure 2, the letters in the circles are single-letter amino acid codes, the connecting lines between chains indicate disulfide bridging, the open circles indicate glycosylation sites, and the designations F, GF, K1, K2, and SP indicate, respectively, the finger, growth factor, kringle 1, kringle 2, and serine protease domains.

10 In Figure 3, the letters in the circles are single-letter amino acid codes, the connecting line between chains indicate disulfide bridging, the open circle indicates a glycosylation site, and the designations GF, K and SP indicate, respectively, the growth factor, kringle, and serine protease domains.

For purposes of shorthand designation of plasminogen activator variants described herein, it is noted that numbers refer to the amino acid residue/position along the amino acid sequences of putative mature t-PA (EPO Publ. No. 93,619), mature human urokinase, and prourokinase. Amino acid identification uses the
15 single-letter alphabet of amino acids, i.e.,

Asp	D	Aspartic acid	Ile	I	Isoleucine
Thr	T	Threonine	Leu	L	Leucine
Ser	S	Serine	Tyr	Y	Tyrosine
Glu	E	Glutamic acid	Phe	F	Phenylalanine
Pro	P	Proline	His	H	Histidine
Gly	G	Glycine	Lys	K	Lysine
Ala	A	Alanine	Arg	R	Arginine
Cys	C	Cysteine	Trp	W	Tryptophan
Val	V	Valine	Gln	Q	Glutamine
Met	M	Methionine	Asn	N	Asparagine

20 The number following such single letters refers to the amino acid position, e.g., D184 means a variant having, *inter alia*, an aspartic acid at position 184.

While the glycosylation site(s) for purposes of this invention may be any site(s) within the molecule that is not already or potentially glycosylated in the corresponding native protein, it is preferably in a position of the molecule that is exposed to the exterior. Such regions include, e.g., amino acid positions 57 to 61, 63 to 69, and 78 to 82 (within the growth factor domain) of t-PA. These regions correspond to loops A, B, and C, respectively, of the type 1 growth factor domains shown in Fig. 2 of Appella et al., *FEBS Letters*, 231: 1-4 (1988). Thus, for O-linked glycosylation, one or more amino acids in these regions is replaced or supplemented with a serine, threonine, or 5-hydroxylysine residue.

Exemplary variants for N-linked glycosylation that employ these regions (where urokinase means human urokinase and also includes human prourokinase) include: S60 t-PA, S17 urokinase, T60 t-PA, T17 urokinase, N64S66 t-PA, N64T66 t-PA, S24 urokinase, T24 urokinase, N65S67 t-PA, N65T67 t-PA, N23S25 urokinase, N23T25 urokinase, N67 t-PA, N67T69 t-PA, N25 urokinase, N25T27 urokinase, N78S80 t-PA, N78T80 t-PA, N36S38 urokinase, N36T38 urokinase, N79S81 t-PA, N79T81 t-PA, N37S39 urokinase, N37T39 urokinase, N80S82 t-PA, N80T82 t-PA, N38S40 urokinase, N38T40 urokinase, or any combination of one loop with another, such as, e.g., S60N65T67 t-PA or S60N78S80 t-PA.

45 In one preferred embodiment, the N-linked glycosylation site is the tyrosine-phenylalanine-serine tripeptidyl sequence at positions 67 to 69 of t-PA, and positions 25 to 27 of human urokinase and human prourokinase.

The plasminogen activator variants herein, in addition to being altered from the native sequence at one or more sites so as to effect glycosylation at those sites where they are not ordinarily glycosylated, also optionally contain substitutions, deletions, or insertions of residues in other regions of the native sequence to improve certain properties of the molecule.

For example, the t-PA variants herein may be devoid of at least a portion of the finger domain, and/or devoid of glycosylation potential at the glycosylation site surrounding amino acid 184, and may exhibit resistance to proteolytic cleavage at the site surrounding amino acids 275 and 276 and/or having amino acid modifications in the putative lysine binding site of kringle 2.

55 In addition, fibrin binding of t-PA can be modulated, most preferably restored or increased, by appropriate substitutions of positively or negatively charged amino acid residues on the opposite edges of the putative ligand binding pocket of t-PA. The variants herein are generally prepared by site-directed

mutagenesis or by excision/ligation techniques described further hereinbelow.

Specific examples of such t-PA variants include a molecule devoid of amino acids 1 to 44 (designated des 1-44), a molecule having aspartic acid at position 184 (designated D184), and a single-chain plasminogen activator mutant. Variants devoid of amino acids 1 to 44 are described more fully in copending U.S. Ser. No. 68,448, *supra*.

All of the above t-PA variants are optionally modified in various other regions of the molecule, for example:

1. Kringle 1 modifications, for example, deletion of about 92 to 179, and/or
2. Kringle 2 modifications, for example, modification in the region of amino acids about 205-215, especially 210-213, and/or
3. Amino acids about 244-255, especially 252 or its site, and/or
4. Amino acids about 233-242, especially 236-238, and/or
5. Known glycosylation sites such as amino acid 184.

Particular embodiments of the above-noted t-PA variants, with asparagine at position 67, are: des 1-44N67D184G275 t-PA, des 1-44N67D184E275 t-PA, des 1-44N67G275 t-PA, des 1-44N67E275 t-PA, des 1-44N67Q275I277 t-PA, des 1-44N67D184E275I277 t-PA, des 1-44N67E275I277 t-PA, des 1-44N67R210A211R212R213E275 t-PA, des 1-44N67R210A211R212R213E275I277 t-PA, des 1-44N67K213E275 t-PA, des 1-44N67K213E275I277 t-PA, des 1-44N67R252E275 t-PA, des 1-44N67R252E275I277 t-PA, des 1-44N67K210E275 t-PA, des 1-44N67K210E275I277 t-PA, des 1-44N67R210H211Q212K213E275 t-PA, des 1-44N67R210H211Q212K213E275I277 t-PA, des 1-44N67D184R210A211R212R213R252E275 t-PA, des 1-44N67D184R210A211R212R213R252E275I277 t-PA, N67-des 92-179D184R210A211R212R213R252E275 t-PA, or the N184 and S184 analogues thereof, and combinations thereof.

Many of these modifications may significantly alter clearance rates and fibrin binding relative to native t-PA. The practitioner skilled in the art will be able to determine by the appropriate assay what the optimum properties of each variant are that are desired in any particular instance.

The modification to change or insert the appropriate amino acid(s) in the native molecule to effect the above sequence variations is accomplished by any means known in the art, such as, e.g., site-directed mutagenesis or ligation of the appropriate sequence into the DNA encoding the relevant protein, as described below.

3. Site-Specific Mutagenesis

Preparation of t-PA variants in accordance herewith is preferably achieved by site-specific mutagenesis of DNA that encodes an earlier prepared variant or a nonvariant version of the protein. Site-specific mutagenesis allows the production of t-PA variants through the use of specific oligonucleotide sequences that encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the junction being traversed. Typically, a primer of about 20 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered. In general, the technique of site-specific mutagenesis is well known in the art as exemplified by publications such as Adelman et al., *DNA*, 2: 183 (1983).

As will be appreciated, the site-specific mutagenesis technique typically employs a phage vector that exists in both a single-stranded and double-stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage, for example, as disclosed by Messing et al., *Third Cleveland Symposium on Macromolecules and Recombinant DNA*, Editor A. Walton, Elsevier, Amsterdam (1981). These phage are readily commercially available and their use is generally well known to those skilled in the art. Alternatively, plasmid vectors that contain a single-stranded phage origin of replication (Veira et al., *Meth. Enzymol.*, 153: 3 (1987)) may be employed to obtain single-stranded DNA.

In general, site-directed mutagenesis in accordance herewith is performed by first obtaining a single-stranded vector that includes within its sequence a DNA sequence that encodes the relevant plasminogen activator. An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically, for example, by the method of Crea et al., *Proc. Natl. Acad. Sci. (USA)*, 75: 5765 (1978). This primer is then annealed with the single-stranded t-PA sequence-containing vector, and subjected to DNA-polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells such as JM101 cells and clones are selected that include recombinant

vectors bearing the mutated sequence arrangement.

After such a clone is selected, the mutated t-PA region is removed and placed in an appropriate vector for t-PA production, generally an expression vector of the type that typically is employed for transformation of an appropriate eukaryotic host. In the context of the present invention, CHO cells or 293 (human kidney cells described by Graham et al., *J. Gen. Virol.*, 36: 59 (1977)) are preferred for the preparation of long-term stable t-PA producers. However, the invention is not limited to CHO production as it is known that numerous other cell types may be employed, particularly where one desires only transient production of the enzyme for test purposes. For example, described below is a transient system employing 293 cells that provides a convenient system for production of t-PA variants for analytical purposes.

4. Cleavage/Ligation Technique

Another method for making mutations in the DNA sequence encoding the plasminogen activator so as to introduce a new glycosylation site involves cleaving the DNA encoding the plasminogen activator at the appropriate position by digestion with restriction enzymes, recovering the properly cleaved DNA, synthesizing an oligonucleotide encoding the desired amino acid sequence for glycosylation and flanking regions such as polylinkers with blunt ends (or, instead of using polylinkers, digesting the synthetic oligonucleotide with the restriction enzymes also used to cleave the activator-encoding DNA, thereby creating cohesive termini), and ligating the synthetic DNA into the remainder of the plasminogen-encoding structural gene.

5. Host Cell Cultures and Vectors

Although Chinese hamster ovary (CHO) expression is ultimately preferred for t-PA production, the vectors and methods disclosed herein are suitable for use in host cells over a wide range of eukaryotic organisms that effect glycosylation of proteins.

In general, of course, prokaryotes are preferred for the initial cloning of DNA sequences and constructing the vectors useful in the invention. For example, *E. coli* K12 strain 294 (ATCC No. 31,446) is particularly useful. Other microbial strains that may be used include *E. coli* strains such as *E. coli* B, and *E. coli* X1776 (ATCC No. 31,537). These examples are, of course, intended to be illustrative rather than limiting.

For expression, eukaryotic hosts, such as yeast and mammalian cultures, are used. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among eukaryotic microorganisms, although a number of other strains are commonly available. For expression in *Saccharomyces*, the plasmid YRp7, for example (Stinchcomb et al., *Nature*, 282: 39 (1979); Kingsman et al., *Gene*, 7: 141 (1979); Tschemper et al., *Gene*, 10: 157 (1980)) is commonly used. This plasmid already contains the *trp1* gene that provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44,076 or PEP4-1 (Jones, *Genetics*, 85: 12 (1977)). The presence of the *trp1* lesion as a characteristic of the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable promoting sequences in yeast vectors include the promoters for 3-phosphoglycerate kinase (Hitzeman et al., *J. Biol. Chem.*, 255: 2073 (1980)) or other glycolytic enzymes (Hess et al., *J. Adv. Enzyme Reg.*, 7: 149 (1968); Holland et al., *Biochemistry*, 17: 4900 (1978)), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. In constructing suitable expression plasmids, the termination sequences associated with these genes are also ligated into the expression vector 3' of the sequence desired to be expressed to provide polyadenylation of the mRNA and termination. Other promoters, which have the additional advantage of transcription controlled by growth conditions, are the promoter region for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and the aforementioned glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Any plasmid vector containing yeast-compatible promoter, origin of replication and termination sequences is suitable.

In addition to eukaryotic microorganisms, cultures of cells derived from multicellular organisms may also be used as hosts. In principle, any such cell culture is workable, whether from vertebrate or invertebrate culture. However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years [*Tissue Culture*, Academic Press, Kruse and Patterson, editors (1973)]. Examples of such useful host cell lines are VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and W138, BHK, COS-7, 293, and MDCK cell lines.

Expression vectors for such cells ordinarily include (if necessary) an origin of replication, a promoter located in front of the gene to be expressed, along with any necessary ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences.

For use in mammalian cells, the control functions on the expression vectors are often provided by viral material. For example, commonly used promoters are derived from polyoma, Adenovirus2, and most frequently Simian Virus 40 (SV40). The early and late promoters of SV40 virus are particularly useful because both are obtained easily from the virus as a fragment that also contains the SV40 viral origin of replication (Fiers et al., *Nature*, 273: 113 (1978). Smaller or larger SV40 fragments may also be used, provided there is included the approximately 250-bp sequence extending from the HindIII site toward the BglII site located in the viral origin of replication. Further, it is also possible, and often desirable, to utilize promoter or control sequences normally associated with the desired gene sequence, provided such control sequences are compatible with the host cell systems.

An origin of replication may be provided either by construction of the vector to include an exogenous origin, such as may be derived from SV40 or other viral (e.g., Polyoma, Adeno, VSV, BPV) source, or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

In selecting a preferred host cell for transfection by the vectors of the invention that comprise DNA sequences encoding both variant t-PA and DHFR protein, it is appropriate to select the host according to the type of DHFR protein employed. If wild-type DHFR protein is employed, it is preferable to select a host cell that is deficient in DHFR, thus permitting the use of the DHFR coding sequence as a marker for successful transfection in selective medium that lacks hypoxanthine, glycine, and thymidine. An appropriate host cell in this case is the CHO cell line deficient in DHFR activity, prepared and propagated as described by Urlaub and Chasin, *Proc. Natl. Acad. Sci. (USA)* 77: 4216 (1980).

On the other hand, if DHFR protein with low binding affinity for MTX is used as the controlling sequence, it is not necessary to use DHFR-deficient cells. Because the mutant DHFR is resistant to methotrexate, MTX-containing media can be used as a means of selection provided that the host cells are themselves methotrexate sensitive. Most eukaryotic cells that are capable of absorbing MTX appear to be methotrexate sensitive. One such useful cell line is a CHO line, CHO-K1 (ATCC No. CCL 61).

Satisfactory amounts of human t-PA are produced by cell cultures; however, refinements, using a secondary coding sequence, serve to enhance production levels even further. The secondary coding sequence comprises dihydrofolate reductase (DHFR) that is affected by an externally controlled parameter, such as methotrexate, thus permitting control of expression by control of the methotrexate (MTX) concentration.

In terms of choosing the best host for the glycosylation, the selection will depend on the type of glycosylation desired, as, for example, yeast hosts will glycosylate differently from mammalian hosts, in terms of the number and types of sugars attached to the protein being expressed.

6. Typical Cloning and Expression Methodology Employable

If cells without formidable cell membrane barriers are used as host cells, transfection is carried out by the calcium phosphate precipitation method as described by Graham and Van der Eb, *Virology*, 52: 546 (1978). However, other methods for introducing DNA into cells such as by nuclear injection or by protoplast fusion may also be used.

If prokaryotic cells or cells that contain substantial cell wall constructions are used, the preferred method of transfection is calcium treatment using calcium as described by Cohen et al., *Proc. Natl. Acad. Sci. (USA)* 69: 2110 (1972).

Construction of suitable vectors containing the desired coding and control sequences employ standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to construct the plasmids required.

Cleavage is performed by treating with restriction enzyme (or enzymes) in suitable buffer. In general, about 1 µg plasmid or DNA fragments is used with about 1 unit of enzyme in about 20 µl of buffer solution. (Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer.) Incubation times of about one hour at 37°C are workable. After incubation, protein is removed by extraction with phenol and chloroform, and the nucleic acid is recovered from the aqueous fraction by precipitation with ethanol.

If blunt ends are required, the preparation may be treated for 15 minutes at 15°C with 10 units of Polymerase I (Klenow), phenolchloroform extracted, and ethanol precipitated.

Size separation of the cleaved fragments is performed using 6 percent polyacrylamide gel described by Goeddel et al., Nucleic Acids Res., 8: 4057 (1980).

For ligation approximately equimolar amounts of the desired components, suitably end tailored to provide correct matching, are treated with about 10 units T4 DNA ligase per 0.5 µg DNA. (When cleaved
5 vectors are used as components, it may be useful to prevent religation of the cleaved vector by pretreatment with bacterial alkaline phosphatase.)

As discussed above, t-PA variants are preferably produced by means of specific mutation. Mutants useful in the practice of the present invention are formed most readily through the use of specific oligonucleotide sequences that encode the DNA sequence of the desired mutation, as well as a sufficient
10 number of adjacent nucleotides, to provide a sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the mutation site being traversed.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures are typically used to transform E. coli K12 strain 294 (ATCC 31,446) or other suitable E. coli strains, and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the
15 transformants are prepared, analyzed by restriction mapping and/or DNA sequencing by the method of Messing et al., Nucleic Acids Res., 9: 309 (1981) or by the method of Maxam et al., Methods of Enzymology, 65: 499 (1980).

After introduction of the DNA into the mammalian cell host and selection in medium for stable transfectants, amplification of DHFR-protein-coding sequences is effected by growing host cell cultures in
20 the presence of approximately 20,000-500,000 nM concentrations of methotrexate, a competitive inhibitor of DHFR activity. The effective range of concentration is highly dependent, of course, upon the nature of the DHFR gene, protein and the characteristics of the host. Clearly, generally defined upper and lower limits cannot be ascertained. Suitable concentrations of other folic acid analogs or other compounds that inhibit DHFR could also be used. MTX itself is, however, convenient, readily available, and effective.

In order to simplify the Examples certain frequently occurring methods will be referenced by shorthand phrases.

"Plasmids" are designated by a low case p followed by an alphanumeric designation. The starting plasmids herein are commercially available, are publicly available on an unrestricted basis, or can be constructed from such available plasmids in accord with published procedures. In addition, other equivalent
30 plasmids are known in the art and will be apparent to the ordinary artisan.

"Digestion" of DNA refers to catalytic cleavage of the DNA with an enzyme that acts only at certain locations in the DNA. Such enzymes are called restriction enzymes, and the sites for which each is specific is called a restriction site. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors and other requirements as established by the enzyme suppliers were used.
35 Restriction enzymes commonly are designated by abbreviations composed of a capital letter followed by other letters representing the microorganism from which each restriction enzyme originally was obtained and then a number designating the particular enzyme. In general, about 1 µg of plasmid or DNA fragment is used with about 2 units of enzyme in about 20 µl of buffer solution. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation times of about 1
40 hour at 37°C are ordinarily used, but may vary in accordance with the supplier's instructions. After incubation, protein is removed by extraction with phenol and chloroform, and the digested nucleic acid is recovered from the aqueous fraction by precipitation with ethanol. Digestion with a restriction enzyme infrequently is followed with bacterial alkaline phosphatase hydrolysis of the terminal 5' phosphates to prevent the two restriction cleaved ends of a DNA fragment from "circularizing" or forming a closed loop
45 that would impede insertion of another DNA fragment at the restriction site. Unless otherwise stated, digestion of plasmids is not followed by 5' terminal dephosphorylation. Procedures and reagents for dephosphorylation are conventional (T. Maniatis et al., Molecular Cloning: A Laboratory Manual, (Cold Spring Harbor Laboratory; New York, 1982), pp. 133-134).

"Recovery" or "isolation" of a given fragment of DNA from a restriction digest means separation of the
50 digest on polyacrylamide or agarose gel by electrophoresis, identification of the fragment of interest by comparison of its mobility versus that of marker DNA fragments of known molecular weight, removal of the gel section containing the desired fragment, and separation of the gel from DNA. This procedure is known generally. For example, see R. Lawn et al., 1981, Nucleic Acids Res. 9:6103-6114, and D. Goeddel et al., 1980, Nucleic Acids Res. 8:4057.

"Southern Analysis" is a method by which the presence of DNA sequences in a digest or DNA-containing composition is confirmed by hybridization to a known, labelled oligonucleotide or DNA fragment. For the purposes herein, unless otherwise provided, Southern analysis shall mean separation of digests on
55 1 percent agarose, denaturation and transfer to nitrocellulose by the method of E. Southern, 1975, J. Mol.

Biol. 98: 503-517, and hybridization as described by T. Maniatis *et al.*, 1978, *Cell* 15: 687-701.

"Transformation" means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or chromosomal integrant. Unless otherwise provided, the method used herein for transformation of *E. coli* is the CaCl_2 method of Mandel *et al.*, 1970, *J. Mol. Biol.* 53: 154.

"Ligation" refers to the process of forming phosphodiester bonds between two double stranded nucleic acid fragments (T. Maniatis *et al.*, *Id.*, p. 146). Unless otherwise provided, ligation may be accomplished using known buffers and conditions with 10 units of T4 DNA ligase ("ligase") per 0.5 μg of approximately equimolar amounts of the DNA fragments to be ligated.

"Preparation" of DNA from transformants means isolating plasmid DNA from microbial culture. Unless otherwise provided, the alkaline/SDS method of Maniatis *et al.*, *Id.*, p. 90, may be used.

"Oligonucleotides" are short length single or double stranded polydeoxynucleotides that are chemically synthesized by known methods and then purified on polyacrylamide gels.

C. Pharmaceutical Compositions

The compounds of the present invention can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby the plasminogen activator product hereof is combined in admixture with a pharmaceutically acceptable carrier vehicle. Suitable carrier vehicles and their formulation, inclusive of other human proteins, e.g., human serum albumin, are described, for example, in *Remington's Pharmaceutical Sciences*, 16th ed., 1980, Mack Publishing Co., edited by Oslo *et al.* Such compositions will typically contain an effective amount of the variant herein, for example, from about 0.5 to about 5 mg/ml, together with a suitable amount of carrier vehicle to prepare pharmaceutically acceptable compositions suitable for effective administration to the host. The plasminogen activator variant herein may be administered parenterally to subjects suffering from cardiovascular diseases or conditions, or by other methods that ensure its delivery to the bloodstream in an effective form.

Compositions particularly well suited for the clinical administration of variant plasminogen activator products employed in the practice of the present invention include, for example, sterile aqueous solutions, or sterile hydratable powders such as lyophilized protein. It is generally desirable to include further in the formulation an appropriate amount of a pharmaceutically acceptable salt, generally in an amount sufficient to render the formulation isotonic. A pH regulator such as arginine base, and phosphoric acid, are also typically included in sufficient quantities to maintain an appropriate pH, generally from 5.5 to 7.5. Moreover, for improvement of shelf-life or stability of aqueous formulations, it may also be desirable to include further agents such as glycerol. In this manner, variant t-PA formulations are rendered appropriate for parenteral administration, and, in particular, intravenous administration.

Dosages and desired drug concentrations of pharmaceutical compositions of the present invention may vary depending on the particular use envisioned. For example, in the treatment of deep vein thrombosis or peripheral vascular disease, "bolus" doses, on the order of about 0.05 to about 0.3 mg/kg, will typically be preferred with subsequent administrations, on the order of about 0.1 to about 0.2 mg/kg, being given to maintain an approximately constant blood level, preferably on the order of about 3 $\mu\text{g}/\text{ml}$.

However, for use in connection with emergency medical care facilities where infusion capability is generally not available and due to the generally critical nature of the underlying disease (e.g., embolism, infarct), it will generally be desirable to provide somewhat larger initial doses, such as an intravenous bolus on the order of about 0.3 mg/kg.

For example, the plasminogen activator variant hereof may be administered parenterally to subjects suffering from cardiovascular diseases or conditions. Dosage and dose rate may be parallel to that currently in use in clinical investigations of other cardiovascular, thrombolytic agents, e.g., about 1-2 mg/kg body weight as an intravenous or intra-arterial dose over 1.5 to 12 hours in human patients suffering from myocardial infarction, pulmonary embolism, etc.

As one example of an appropriate dosage form, a vial containing 50 mg t-PA, arginine, phosphoric acid, and polysorbate 80 may be reconstituted with 50 ml sterile water for injection and mixed with a suitable volume of 0.9 percent sodium chloride injection.

The extended half-life of plasminogen activator variants herein may be suitable for rapid intravenous injection, particularly as a bolus, for example. This would eliminate the need for complex administration procedures and may increase the opportunity for the use of the plasminogen activator in settings with limited medical equipment such as in emergency vehicles staffed with paramedic personnel. An extended half-life of plasminogen activator variants herein may also allow lower, safer initial doses and could maintain thrombolytically effective plasma levels for up to 45 minutes or longer. The variants herein with extended half-life may also be useful for low-dose extended therapy that may be necessary to avoid reocclusion

following successful acute thrombolysis or for extended thrombolysis that may be necessary in cases of peripheral vascular occlusion.

The following examples are intended merely to illustrate the best mode now known for practicing the invention, but the invention is not to be considered limited thereto.

5

EXAMPLE I

A. Preparation and Utilization of Expression Vectors for Recombinant Production of the t-PA Variants Hereof

10 1. Construction of Plasmid p7-1H

a) Plasmid pCIS-t-PA

Plasmid pCIS-t-PA was prepared as described, for example, in EPO Publ. 199,475, published 29
15 October 1986, *supra*. In recapitulation, the vector pCIS-t-PA containing the cytomegalovirus enhancer and promoter, the cytomegalovirus splice donor site and intron, the Ig variable region splice acceptor site, the cDNA-encoding t-PA (Pennica et al., *Nature*, 301: 214 (1983)) and the hepatitis surface antigen polyadenylation and transcription termination site was constructed first:

The vector pF8CIS containing the cytomegalovirus enhancer (Boshart et al., *Cell*, 41: 520 (1985)) and
20 promoter (Thomsen et al., *Proc. Natl. Acad. Sci. (U.S.A.)* 81: 659 (1984)), the cytomegalovirus splice donor site and a portion of an intron (Sternberg et al., *J. of Virol.*, 49: 190 (1984)), the Ig variable region intron and splice acceptor site, the cDNA encoding factor VIII, and the SV40 polyadenylation site was constructed. The three parts of the construction are detailed below.

1. The ampicillin resistance marker and replication origin of the final vector was derived from the starting
25 plasmid pUC13pML, a variant of the plasmid pML (Lusky et al., *Nature*, 293: 79 (1981)). pUC13pML was constructed by transferring the polylinker of pUC13 (Veira et al., *Gene*, 19: 259 (1982)) to the *EcoRI* and *HindIII* sites of pML. A second starting plasmid pUC8CMV was the source of the CMV enhancer, promoter and splice donor sequence. pUC8CMV was constructed by inserting nucleotides 1 through 732 for the CMV enhancer, promoter and splice donor sequence into the blunted *PstI* and *SphI* sites of pUC8
30 - Veira et al., *supra*. Synthetic *BamHI-HindIII* linkers (commercially available from New England Biolabs) were ligated to the cohesive *BamHI* end, creating a *HindIII* site. Following this ligation a *HindIII-HincII* digest was performed. This digest yielded a fragment of approximately 800 bp that contained the CMV enhancer, promoter and splice donor site. Following gel isolation, this 800-bp fragment was ligated to a 2900-bp piece of pUC13pML. The fragment required for the construction of pF8CIS was obtained by
35 digestion of the above intermediate plasmid with *SalI* and *HindIII*. This 3123-bp piece contained the resistance marker for ampicillin, the origin of replication from pUC13pML, and the control sequences for the CMV, including the enhancer, promoter, and splice donor site.

2. The Ig variable region intron and splice acceptor sequence was constructed using a synthetic
40 oligomer. A 99-mer and a 30-mer were chemically synthesized having the following sequence for the IgG intron and splice acceptor site (Bothwell et al., *Cell*, 24: 625 (1981)):

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1      5' -AGTAGCAAGCTTGACGTGTGGCAGGCTTGA...
45    31      GATCTGGCCATACACTTGAGTGACAATGA...
      60      CATCCACTTTGCCTTTCTCTCCACAGGT...
      88      GTCCACTCCCAG-3'
50    1      3' -CAGGTGAGGGTGCAGCTTGACGTCGTCGGA-5'

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DNA polymerase I (Klenow fragment) filled in the synthetic piece and created a double - stranded
fragment (Wartell et al., *Gene*, 9: 307 (1980)). This was followed by a double digest of *PstI* and *HindIII*.
This synthetic linker was cloned into pUC13 (Veira et al., *supra*) at the *PstI* and *HindIII* sites. The clone
55 containing the synthetic oligonucleotide, labeled pUCIg.10, was digested with *PstI*. A *ClaI* site was added to this fragment by use of a *PstI-ClaI* linker. Following digestion with *HindIII* a 118- bp piece containing part of the Ig intron and the Ig variable region splice acceptor was gel isolated.

3. The third part of the construction scheme replaced the hepatitis surface antigen 3' end with the polyadenylation site and transcription termination site of the early region of SV40. A vector, pUC.SV40, containing the SV40 sequences was inserted into pUC8 at the BamHI site described in Veira et al., supra. pUC.SV40 was then digested with EcoRI and HpaI. A 143-bp fragment containing only the SV40 polyadenylation site was gel isolated from this digest. Two additional fragments were gel isolated following digestion of pSVE.8c1D (EPO Pub. No. 160,457). The 4.8-kb fragment generated by EcoRI and Clal digest contains the SV40-DHFR transcription unit, the origin of replication of pML, and the ampicillin resistance marker. The 7.5-kb fragment produced following digestion with Clal and HpaI contains the cDNA for Factor VIII. A three-part ligation yields pSVE.8c24D. This intermediate plasmid was digested by Clal and SalI to give a 9611-bp fragment containing the cDNA for Factor VIII with the SV40 polyadenylation and transcription termination sites followed by the SV40 DHFR transcription unit.

The final three-part ligation to yield pF8CIS used: a) the 3123-bp SalI-HindIII fragment containing the origin of replication, the ampicillin resistance marker and the CMV enhancer, promoter and splice donor; b) the 118-bp HindIII-Clal fragment containing the Ig intron and splice acceptor; and c) a 9611-bp Clal-SalI fragment containing the cDNA for Factor VIII, SV40 polyadenylation site, and the SV40 DHFR transcription unit.

Next, the completion of the construction of plasmid pCIHt-PA from intermediate plasmid pClat-PA and plasmid pF8CIS (above) was undertaken:

The t-PA cDNA was first cloned into pML to provide a Clal site at the 5' end of the gene. To do this, a 3238-bp HindIII fragment from pSVpa-DHFR (otherwise referred to as pETPFR, supra) was inserted into the HindIII site of pML (Lusky et al., supra). Colonies were screened for clones that have the 5' end of the cDNA juxtaposed to the Clal site. The intermediate plasmid was labeled pCLat-PA. A t-PA cDNA followed by the 3'- polyadenylation regions was isolated as a Clal-KpnI fragment of 2870 bp. This fragment was ligated to the 5146-bp fragment of pF8CIS. This Clal-KpnI fragment of the CIS vector provided the 5' control region, a SV40-DHFR transcriptional unit, the ampicillin resistance gene, and the origin region from pML. See Figures 4a and 4b.

Expression levels of t-PA were obtained by transfecting CHO or 293 cells with pCIHt-PA, in accordance with methods generally known per se and described supra. Media from the transfected 293 cells, for example, were assayed, demonstrating that pCIHt-PA produced 420 ng/ml of t-PA.

The vector pCIS-t-PA containing the cytomegalovirus enhancer and promoter, the cytomegalovirus splice donor site and intron, the Ig variable region splice acceptor site, the cDNA encoding t-PA, and the pSV40 polyadenylation sequence was finally constructed as follows:

The starting vectors for this construction were pCIHt-PA and pF8CIS (supra). The latter vector has the same 5' controls as pCIHt-PA, but includes the cDNA for Factor VIII and the SV40 polyadenylation site. SacII was used to cleave 3' of the t-PA cDNA. The resultant 3' overhang was blunted by T4 polymerase. pCIHt-PA was then cut with Clal. This site separates the chimeric intron cleaving between the CMV intronic sequences and the Ig variable region intron. An 2870-bp fragment was gel isolated from the Clal treatment. The SV40 polyadenylation site, DHFR, transcription control, bacterial origin of replication, and amp^r gene, as well as the CMV enhancer and promoter and splice donor were isolated from pF8CIS. These elements were isolated into fragments as a 2525-bp Sal-Clal fragment and a HpaI-Sal and 3113-bp fragment. A three-part ligation of the Kpnl (blunt)-Clal fragment with the HpaI-Sal fragment and Sal to BamHI fragment yields pCIS-t-PA, which was expressed in both CHO and 293 cells as discussed above for plasmid pCIHt-PA, giving 55 and 3000 ng/ml of t-PA, respectively. See Figures 5a and 5b.

b) Final Construction of p7-1H

The plasmid pCIS-t-PA was digested with SpeI, then treated with E. coli DNA polymerase I large fragment (Klenow) and deoxyribonucleoside triphosphates to create blunt ends. The resulting linear fragment was ligated, using T4 DNA ligase, to the 0.45kb-RsaI/AhaIII fragment containing the + strand origin from the single-stranded DNA phage, f1, as described in Zinder et al., Microbiol. Rev., 49: 101 (1985). Ligation products were isolated with the f1 origin inserted in both possible orientations at the SpeI site of the pCIS-t-PA fragment. A plasmid containing this origin, in such an orientation that the anti-sense strand of the t-PA gene was packaged into virions in the presence of helper phage, was chosen and termed p7-1H. See Figure 6.

2. Mutagenesis Examples

a) Template Preparation

5 Plasmid p7-1H was introduced into E. coli strain JM101 (ATCC No. 33,876) via CaCl_2 -mediated transformation. These cells were then infected with the helper virus M13K07 and single-stranded p7-1H DNA was prepared as described by Veira et al., Meth. Enzymol., 153: 3 (1987). Briefly, to 0.3 ml of a saturated culture of transformed cells in 2YT broth was added 10^9 - 10^{10} pfu of M13K07 and the mixture was incubated for 15 min. at 37°C . 1.5 ml of fresh 2YT broth, containing 50 $\mu\text{g/ml}$ carbenicillin, was added and
 10 the culture was gently shaken for 16 hours at 37°C . After the cells were pelleted, phage and packaged plasmid DNA were harvested, and single-stranded DNA was prepared as described by Anderson, Nucl. Acids. Res., 9: 3015 (1981).

b) Site-directed in vitro Mutagenesis

15 Mutagenesis on p7-1H was carried out using the oligodeoxyribonucleotide, 5-CAGCAGGCCCTGAATTTCTCAG-3', essentially as described by Zoller et al., Meth. Enzymol., 100: 468 (1983), except that the mutant, with the mutation Tyr67 $\rightarrow$ Asn67, was identified by colony hybridization rather than plaque hybridization. Mutations were verified by DNA sequencing directly on the single-stranded
 20 plasmid DNA using the dideoxynucleotide chain termination method (Sanger et al., Proc. Natl. Acad. Sci. - (U.S.A.) 74: 5463 (1977)).

3. Expression and Purification

25 a) Plasmid Preparation

Transformed cells were grown to saturation in 500-ml LB broth containing 50 $\mu\text{g/ml}$ carbenicillin. Cells were pelleted by centrifugation and resuspended in 40 ml of 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl (pH 8.0). To this suspension was added 60 ml of 1% sodium dodecyl sulfate, 0.07 M NaOH, and the
 30 mixture was incubated for 2 min at 25°C , then at 10 min. at 0°C . To this 52 ml of 4 M acetic acid, 3 M sodium acetate was added and the mixture was incubated for 30 min. at 0°C . This was then centrifuged at 11,500 rpm for 20 min., the supernatant mixed with two volumes of 100% cold ethanol, and the resulting precipitate harvested by centrifugation. The pellet, containing plasmid DNA and RNA, was dried and redissolved in 100 mM Tris (pH 8.0), 10 mM EDTA, 1 $\mu\text{g/ml}$ RNase A. After the resulting solution was
 35 clarified by centrifugation, it was adjusted to 0.5 mg/ml in ethidium bromide and an equal weight of CsCl was added. The DNA was then centrifuged in a Beckman VTi65 rotor for 16 hours at 55,000 rpm at 18°C . The DNA band was harvested by side puncture, extracted with n-butanol to remove the ethidium bromide, diluted with H_2O , and precipitated by ethanol. DNA was redissolved in 10 mM Tris (pH 8.0), 1 mM EDTA, to a final concentration of 1 mg/ml.

40

b) Transfection and Expression

293 cells were grown to confluence. Ten μg of t-PA mutant plasmid DNA was mixed with 1 μg of DNA encoding the VA RNA gene (Thimmappaya et al., Cell, 31: 543 (1982)) and dissolved in 500 μl of 1 mM
 45 Tris-HCl, 0.1 mM EDTA, 0.227 M CaCl_2 . Added to this (dropwise while vortexing) was 500 μl of 50 mM HEPES (pH 7.35), 280 mM NaCl, 1.5 mM NaPO_4 , and the precipitate was allowed to form for 10 min. at 25°C . The suspended precipitate was then added to the cells (in 100 mM plate) and allowed to settle for four hours in the incubator. The medium was then aspirated off and 2 ml of 20% glycerol in phosphate-buffered saline was added for 30 sec. The cells were washed twice with 5 ml of serum-free medium, then
 50 fresh medium was added and the cells were incubated for five days.

For the creation of stable CHO cell lines expressing the t-PA variant, the BglII/ApaI fragment containing the bulk of the t-PA coding sequences (Figure 6) is ligated to the 6.0-kb BglII/ApaI fragments from the vector pPADHFR-6 (described in EPO Pat. Publ. No. 93,819). The resultant plasmid is then introduced into
 55 CHO cells and induced to over-express the t-PA variant by amplifying the coding sequence by means of selection in methotrexate-containing media.

c) Purification

Purification of the t-PA product was accomplished by passing the conditioned medium over a column (1-ml bed volume) of controlled glass beads to which an anti-t-PA goat polyclonal A6 antibody (prepared according to standard methods known per se) had been coupled. Before the medium was loaded, the column was equilibrated with phosphate-buffered saline and, after loading, the column was equilibrated with 0.1 M Tris-HCl (pH 7.5), 1 M NaCl. The t-PA was eluted with 0.1 M acetic acid, 0.15 M NaCl, 0.02 M arginine, 0.01% Tween 80 (pH 2.0), and fractions were immediately neutralized with Tris-base. Fractions were adjusted to 0.01% Tween 80 before pooling.

B. Biological and Pharmacokinetic Assays

1. t-PA Quantitation

Protein concentrations were routinely determined by an ELISA standardized to native-sequence t-PA (See EPO Pat. Publ. 93,619, supra). Protein purity and homogeneity were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (PAGE-SDS) with the buffer system of Laemmli, Nature, 227: 680 (1970). Typically, 7 to 17% gradient gels were used and proteins were visualized with the silver-staining technique of Morrissey, Anal. Biochem., 117: 307 (1981). The N67 t-PA variant prepared as described above was found to be pure and homogeneous by this method.

2. S-2251 Assay

The ability of t-PA to activate plasminogen can be measured in an in vitro assay by preincubating t-PA and plasminogen and then adding the plasmin-specific substrate H-D-valyl-H-leucyl-H-lysine-p-nitronilide (S-2251). The maximum rate of this reaction is observed in the presence of fibrin(ogen) or fragments of fibrin(ogen) that act as stimulators of the reaction.

The plasmin-specific substrate S-2251 was used in a two-stage assay to measure the ability of the sample to activate plasminogen. Fibrinogen could be used as a stimulator by incubating the sample with 0.02 ml of a 20 mg/ml fibrinogen solution in a total volume of 0.12 ml of 0.05 M Tris-HCl, 0.12 M NaCl, 0.01% Tween 80, pH 7.4.

Glu-plasminogen solution (commercially available), 0.03 ml of a 2.0 mg/ml solution in 0.05M Tris, 0.12 M NaCl buffer, pH 8, was then added. After ten min. at 37 °C, 0.35 ml of 0.86 mM S-2251 in 0.037 M Tris, 0.086 M NaCl, 0.007% Tween 80, pH 7.4 was added. This mixture was incubated for five minutes; then the reaction was stopped by the addition of 0.1 ml of 50% glacial acetic acid. Absorbance at 405 nm was measured. The activity was expressed as the change in absorbance per nanogram per minute in the presence of substrate.

The assay was run as described, along with an additional set of samples that did not contain fibrinogen. The stimulation is the ratio of the specific activity of the sample containing fibrinogen to the specific activity of the sample not containing fibrinogen. The results, along with clot lysis, are shown in Figure 7 for both rt-PA and N67 t-PA. Because the specific activity in vitro of the N67 t-PA appears to be compromised more significantly in the absence of fibrinogen, the percent fibrinogen stimulation (which reflects the ratio of the activity in the presence of fibrinogen to the activity in the absence of fibrinogen) is slightly increased over the wild-type rt-PA.

3. Clot Lysis

Wild-type and N67 t-PA were assayed for their ability to lyse fibrin in the presence of saturating concentrations of plasminogen, according to the method of Carlsen et al., Anal. Biochem., 168: 428 (1988). The in vitro clot lysis assay measures the activity of tissue plasminogen activators by turbidimetry using a microcentrifugal analyzer. A mixture of thrombin and t-PA test samples is centrifuged into a mixture of fibrinogen and plasminogen to initiate clot formation and subsequent clot dissolution. The resultant profile of absorbance versus time is analyzed to determine the assay endpoint. Activities of the t-PA variants were compared to a standard curve of rt-PA (EPO Publ. No. 93,619, supra). The buffer used throughout the assay was 0.06 M sodium phosphate, pH 7.4, containing 0.01% (v/v) Tween 80 and 0.01% (w/v) sodium azide. Human thrombin was at a concentration of 33 units/ml. Fibrinogen (at 2.0 mg/ml clottable protein) was chilled on wet ice to precipitate fibronectin and then gravity filtered. Glu-plasminogen was at a concentration of 1 mg/ml. The analyzer chamber temperature is set at 37 °C The loader is set to dispense

20 μ l of rt-PA (about 500 ng/ml to 1.5 μ g/ml) as the sample for the standard curve, or 20 μ l of variant rt-PA at a concentration to cause lysis within the range of the standard curve. Twenty μ l of thrombin was used as the secondary reagent, and 200 μ l of a 50:1 (v/v) fibrinogen: plasminogen mixture as the primary reagent. The absorbance/time program was used with a five-minute incubation time, 340- μ m filter, and 90-interval readings.

The results, shown in Figure 7, indicate that the N67 variant has about 53% of the clot lysis specific activity of normal wild-type t-PA.

4. Fibrin Binding

The method for fibrin binding is a modification of the method described by Rijken et al., *J. Biol. Chem.*, 257: 2920 (1982). The t-PA sample to be tested is added to a solution containing 0.05 M Tris (pH 7.4), 0.12 M NaCl, 0.01% Tween 80, 1 mg/ml human serum albumin, and various concentrations of plasminogen-free fibrin (0, 0.05, 0.1, 0.25, and 0.5 mg/ml). The final volume of the reaction mixture was 1 ml. The sample was incubated at 37°C for 5 min., followed by the addition of 1 unit of thrombin. The samples were then incubated for one hour at 37°C. The clot was removed by centrifugation, and the amount of t-PA remaining unbound in the supernatant was determined by ELISA.

The data, shown in Figure 8, are plotted as percent t-PA variant bound versus the fibrin(ogen) concentrations for both N67 t-PA and rt-PA. The results indicate that the N67 t-PA does not bind to fibrin under the assay conditions employed.

5. Verification of Glycosylation

The determination of extra glycosylation at asparagine 67 was done by a tryptic mapping method that utilizes high performance liquid chromatography (HPLC) to resolve a mixture of tryptic peptides into individual components or peaks, whose identities and composition are known. The addition of carbohydrate to a peptide causes an increase in hydrophilicity of that peptide and consequently a change in the HPLC profile (i.e., earlier elution). Individual chromatographic peaks containing the isolated peptide were collected and subjected to amino acid analysis to confirm the identity of the peptide and presence of carbohydrate.

a. Sample Preparation. The method of sample preparation for tryptic mapping was a modification of Crestfield, Stein and Moore, *J. Biol. Chem.* 238: 622 (1963). The protein samples to be analyzed (1.0 mg each) were dialyzed overnight into 8 M urea, 0.5 M Tris at pH 8.6 with 2 mM EDTA. The samples were reduced with 10 mM dithiothreitol (Sigma Chemical Co.) for 2 hours at 25°C, then S-carboxymethylated with 25 mM iodoacetic acid (Sigma Chemical Co.) at 25°C in the dark. After 30 minutes the alkylation reaction was quenched by the addition of 20 mM dithiothreitol; then the reduced and carboxymethylated (RCM) samples were dialyzed overnight in 100 mM ammonium bicarbonate at pH 8.3 using dialysis tubing with a 3500 molecular weight cut-off.

b. Treatment with N-Glycosidase F. Reduced and carboxymethylated rt-PA was deglycosylated with N-Glycosidase F (Genzyme Corp.) before trypsin digestion. An aliquot (0.4 mg) of reduced and carboxymethylated rt-PA was reconstituted in 0.08 mL of 250 mM sodium phosphate pH 8.6 containing 10 mM EDTA and 0.02 percent sodium azide. N-Glycosidase F (5.0 Manufacturer's Units in 0.018 mL of 50 percent glycerol) was added to the sample, which was then incubated overnight at 37°C. The sample was diluted to 0.4 mL with water and dialyzed against 100 mM ammonium bicarbonate prior to trypsin digestion.

c. Tryptic Digestion. RCM rt-PA was digested in 0.1 M ammonium bicarbonate at ambient temperature with an addition of [L-(tosylamido-2-phenyl)ethylchloromethylketone] (TPCK)-treated trypsin (Cooper Biomedical) at an enzyme-to-substrate ratio of 1:100 (w/w), followed by a second addition of 1:100 after eight hours. The digestions were stopped after 24 hours by freezing (-70°C).

d. Chromatography. The HPLC separations were performed with a Hewlett-Packard 1090 M liquid chromatograph using a 0.4 x 15 cm Nova PAK, 5 micron, C-18 reverse phase column (Waters, Inc.). The elution profile was monitored for dual wavelength detection at 214 and 280 nanometers. A trifluoroacetic acid (TFA) solvent system was used, employing 0.1 percent TFA (Pierce Chemical) with a linear gradient of 0.08 percent TFA in acetonitrile (Burdick & Jackson) at a rate of 0.5 percent per minute for 50 minutes, followed by a 1.0 percent per minute linear gradient for 35 minutes at a flow rate of 1.0 milliliters per minute.

e. Amino Acid Analysis. Peptide peaks collected from HPLC were characterized by amino acid analysis after acid hydrolysis. Hydrolysis was performed by incubation of the peptides in constant boiling HCl for 20 hours at 110°C in vacuo. Analysis of the acid hydrolyzates was accomplished with a Beckman 6300

amino acid analyzer.

f. **Results.** In Figure 9, the HPLC profiles of tryptic digests are shown for wild-type RCM rt-PA, N67 mutant RCM t-PA and N-glycanase-treated N67 mutant RCM t-PA in Figures 9A, 9B, and 9C, respectively. All samples were produced by transient expression in 293 human kidney cells. The elution profiles seen in Figures 9A-9C are taken from that segment of the gradient where tryptic peptide (56-82) is found to elute for wild-type tPA. This peptide (56-82) eluted as a partially resolved doublet at 55 minutes as indicated by the arrow in Figure 9A.

The N67 tPA mutant was examined in similar fashion (Fig 9B) and was shown to have lost the wild-type tryptic peptide (56-82) that had eluted at 55 minutes. There appeared a corresponding increase in a broad peak at 51 minutes as shoulder on a pre-existing peak (50.5 minutes). The new peak at 51 minutes was collected, acid hydrolyzed, and quantitated by amino acid analysis. The analysis indicated the presence of amino-sugar containing peptide, which was consistent with the earlier elution of glycosylated peptide (56-82).

For confirmation of the glycosylation of the (56-82) tryptic peptide, the RCM N67 t-PA mutant was treated with N-glycosidase to remove N-linked carbohydrate moieties. The tryptic digest of the glycanase-treated mutant protein was analyzed by reverse phase HPLC (Fig. 9C). The earlier eluting shoulder at 51 minutes disappeared with N-glycanase treatment and a new peak at 54.5 minutes appeared in the tryptic map. The slightly earlier elution of N67 (56-82) at 54.5 minutes compared to the wild-type tryptic peptide (56-82) at 55 minutes was consistent with the substitution of tyrosine for aspartic acid (Guo et al., J. Chrom., 359: 499-517, (1986)). (Although the N67 mutant contains carbohydrate linked to asparagine, after N-glycanase treatment the asparagine is converted to an aspartic acid residue.) The assignment of the peak in Figure 9C at 54.5 minutes as peptide (56-82) was confirmed by amino acid analysis. As seen in Table I the observed amino acid composition follows closely the expected composition, confirming the substitution of tyrosine for asparagine and the N-linked glycosylation of that residue at position 67.

TABLE I

Amino Acid Composition of De-glycosylated Tryptic Peptide (N67: 56-82)		
Amino Acid		Expected Observed
Carboxymethyl Cysteine ^a	4	3.4
Aspartate/Asparagine	3	3.0
Threonine	1	1.0
Glutamate/Glutamine	4	4.0
Proline/Cysteic acid ^a	1	1.3
Glycine	4	3.8
Alanine	2	2.1
Half-Cystine ^a	0	0.2
Valine	1	1.1
Methionine	0	0.1
Isoleucine	0	0.3
Leucine	1	1.4
Tyrosine	0	0.1
Phenylalanine	4	3.8
Histidine	0	0.2
Lysine	1	1.0
Arginine	0	0.2

^a The values of carboxymethylated cysteine, cysteic acid and half cystine add to 3.9 compared to the expected value of 4.0.

6. Pharmacokinetics

Twenty rabbits were assigned randomly to one of two treatment groups: rt-PA and glycosylated N67 t-PA. The proteins were labeled with ¹²⁵I to approximately 10 μ Ci/ μ g and mixed with 0.1 mg/kg rt-PA to decrease nonspecific adsorption of the labeled protein. The dose of TCA precipitable ¹²⁵I-protein was nominally 5 μ Ci/kg.

The rabbits had a catheter with a heparin lock in each ear. The dose was administered as an IV bolus in one catheter, followed by a saline flush. All blood samples were obtained from the opposite ear. One ml blood samples were obtained at the following times: 0 (before the dose) and 2, 5, 15, 30, 45, 60, 75, 90, 120, 150, and 180 minutes after the dose. Saline was used to flush the catheters and replace blood volume at each time point. The blood samples were put into 1.5-ml Eppendorf tubes containing 4.2 mM EDTA and 1 mM PPACK (a peptide of phenylalanine-proline-arginine-chloromethyl ketone). The tubes were maintained on ice until centrifuged. After centrifugation, the plasma was removed immediately, placed in Eppendorf tubes, and stored on ice until the end of the study. Proteins in 100 μ l of each plasma sample were precipitated with trichloroacetic acid. The 125 I that was bound to proteins was quantified by counting the gamma emissions of each precipitate. The results were based on CPM/100 μ l of sample and converted to CPM/ml for data analysis.

The area under the curve (AUC) for each rabbit was computed from 2 to 180 minutes by the trapezoid method using the AUC procedure. Clearance was calculated from the formula $CL = \text{Dose}/\text{AUC}$.

The ranking of terminal half-lives for the 125 I-labeled proteins is as follows: rt-PA, N67 t-PA. The actual half-life values must be determined from pharmacokinetic studies with unlabeled proteins. Figure 10 shows the plots of CPM/ml versus minutes (normalized by dose) for rt-PA and glycosylated N67 t-PA, as well as for des 1-44 t-PA, des 1-44E275 t-PA, and des 1-44E275D184 t-PA, for comparison.

The N67 t-PA was cleared more slowly than the wild-type rt-PA from plasma. The ratio of the clearance of the N67 t-PA to wild-type rt-PA was 0.63. Consequently, at equal infusion rates one would have a 1.6-fold higher plasma concentration with the N67 t-PA compared to wild-type t-PA.

Claims

1. A fibrinolytically active human tissue plasminogen activator (t-PA) amino acid sequence variant that has N-linked or O-linked glycosylation at a glycosylation site which is not present in native human t-PA, said plasminogen activator variant exhibiting increased half-life and decreased clearance rate relative to native human t-PA.
2. The variant of claim 1 wherein said glycosylation is an N-linked glycosylation.
3. The variant of claim 2 wherein an N-linked glycosylation site is added in a position of the t-PA variant that is exposed to the exterior.
4. The variant of anyone of claims 1 to 3 that is glycosylated within its growth factor domain.
5. The variant of claim 1 that contains (1) a serine or threonine at position 60 of the native t-PA, (2) an asparagine at position 64 and a serine or threonine at position 66 of the native t-PA, (3) an asparagine at position 65 and a serine or threonine at position 67 of the native t-PA, (4) an asparagine at position 67 of the native t-PA, (5) an asparagine at position 78 and a serine or threonine at position 80 of the native t-PA, (6) an asparagine at position 79 and a serine or threonine at position 81 of the native t-PA, (7) an asparagine at position 80 and a serine or threonine at position 82 of the native t-PA, or (8) a combination of any two or more of (1) to (7) above.
6. The variant of claim 5 wherein the variant contains an asparagine at position 67 of the native t-PA.
7. The variant of claim 4 that is modified by functional removal of at least a portion of a domain responsible for fibrin binding but that has restored fibrin binding comparable to that of native t-PA.
8. The variant of claim 4 that is devoid of at least a portion of the finger domain.
9. The variant of claim 8 that comprises natural t-PA devoid of amino acids 1-44.
10. The variant of any one of claims 1 to 8 modified at a known glycosylation site of native human t-PA.
11. The variant of claim 10 devoid of functional carbohydrate structure at amino acid 184.
12. The variant of claim 11 still further modified, relative to native t-PA, in the amino acid (1) 205 to 215 region, (2) 244 to 255 region, (3) 233-242 region, or (4) two or more of (1), (2) and (3).



The rabbits had a catheter with a heparin lock in each ear. The dose was administered as an IV bolus in one catheter, followed by a saline flush. All blood samples were obtained from the opposite ear. One ml blood samples were obtained at the following times: 0 (before the dose) and 2, 5, 15, 30, 45, 60, 75, 90, 120, 150, and 180 minutes after the dose. Saline was used to flush the catheters and replace blood volume at each time point. The blood samples were put into 1.5-ml Eppendorf tubes containing 4.2 mM EDTA and 1 mM PPACK (a peptide of phenylalanine-proline-arginine-chloromethyl ketone). The tubes were maintained on ice until centrifuged. After centrifugation, the plasma was removed immediately, placed in Eppendorf tubes, and stored on ice until the end of the study. Proteins in 100 μ l of each plasma sample were precipitated with trichloroacetic acid. The 125 I that was bound to proteins was quantified by counting the gamma emissions of each precipitate. The results were based on CPM/100 μ l of sample and converted to CPM/ml for data analysis.

The area under the curve (AUC) for each rabbit was computed from 2 to 180 minutes by the trapezoid method using the AUC procedure. Clearance was calculated from the formula $CL = \text{Dose}/\text{AUC}$.

The ranking of terminal half-lives for the 125 I-labeled proteins is as follows: rt-PA, N67 t-PA. The actual half-life values must be determined from pharmacokinetic studies with unlabeled proteins. Figure 10 shows the plots of CPM/ml versus minutes (normalized by dose) for rt-PA and glycosylated N67 t-PA, as well as for des 1-44 t-PA, des 1-44E275 t-PA, and des 1-44E275D184 t-PA, for comparison.

The N67 t-PA was cleared more slowly than the wild-type rt-PA from plasma. The ratio of the clearance of the N67 t-PA to wild-type rt-PA was 0.63. Consequently, at equal infusion rates one would have a 1.6-fold higher plasma concentration with the N67 t-PA compared to wild-type t-PA.

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1. A fibrinolytically active human tissue plasminogen activator (t-PA) amino acid sequence variant that has N-linked or O-linked glycosylation at a glycosylation site which is not present in native human t-PA, said plasminogen activator variant exhibiting increased half-life and decreased clearance rate relative to native human t-PA.
2. The variant of claim 1 wherein said glycosylation is an N-linked glycosylation.
3. The variant of claim 2 wherein an N-linked glycosylation site is added in a position of the t-PA variant that is exposed to the exterior.
4. The variant of anyone of claims 1 to 3 that is glycosylated within its growth factor domain.
5. The variant of claim 1 that contains (1) a serine or threonine at position 60 of the native t-PA, (2) an asparagine at position 64 and a serine or threonine at position 66 of the native t-PA, (3) an asparagine at position 65 and a serine or threonine at position 67 of the native t-PA, (4) an asparagine at position 67 of the native t-PA, (5) an asparagine at position 78 and a serine or threonine at position 80 of the native t-PA, (6) an asparagine at position 79 and a serine or threonine at position 81 of the native t-PA, (7) an asparagine at position 80 and a serine or threonine at position 82 of the native t-PA, or (8) a combination of any two or more of (1) to (7) above.
6. The variant of claim 5 wherein the variant contains an asparagine at position 67 of the native t-PA.
7. The variant of claim 4 that is modified by functional removal of at least a portion of a domain responsible for fibrin binding but that has restored fibrin binding comparable to that of native t-PA.
8. The variant of claim 4 that is devoid of at least a portion of the finger domain.
9. The variant of claim 8 that comprises natural t-PA devoid of amino acids 1-44.
10. The variant of any one of claims 1 to 8 modified at a known glycosylation site of native human t-PA.
11. The variant of claim 10 devoid of functional carbohydrate structure at amino acid 184.
12. The variant of claim 11 still further modified, relative to native t-PA, in the amino acid (1) 205 to 215 region, (2) 244 to 255 region, (3) 233-242 region, or (4) two or more of (1), (2) and (3).



13. The, variant of claim 4 that as compared with native t-PA is resistant to specific enzymatic cleavage into two-chain form.
14. The variant of claim 13 essentially free of two-chain form.
- 5 15. The variant of claim 14 that is stabilized in single-chain form by site-directed mutagenesis at a two-chain cleavage site.
- 10 16. The variant of claim 15 wherein position 275 numbered in accordance with the mature plasminogen activator is occupied by an amino acid other than arginine.
17. The variant of claim 16 in which said amino acid is selected from glycine and glutamic acid.
18. The variant of claim 17 in which said amino acid is glutamic acid.
- 15 19. The variant of claim 16 wherein position 277 numbered in accordance with the mature plasminogen activator occupied by an amino acid other than lysine.
- 20 20. The variant of claim 19 in which said amino acid is isoleucine.
21. The variant of claim 9 that is resistant to enzymatic cleavage at the amino acid position 275 or the 277 site or both.
22. The variant of claim 21 selected from
25 des 1-44N67E275 t-PA,
des 1-44N67D184E275 t-PA,
des 1-44N67S184E275 t-PA,
des 1-44N67K213E275 t-PA,
des 1-44N67R210A211R212R213E275 t-PA,
30 des 1-44N67R252E275 t-PA,
des 1-44N67K210E275 t-PA,
des 1-44N67E275I277 t-PA,
des 1-44N67D184E275I277 t-PA,
des 1-44N67S184E275I277 t-PA,
35 des 1-44N67K213E275I277 t-PA,
des 1-44N67R210A211R212R213E275I277 t-PA,
des 1-44N67R252E275I277 t-PA, and
des 1-44N67K210E275I277 t-PA.
- 40 23. A DNA sequence encoding the variant of any one of claims 1 to 11 and 16 to 22.
24. A replicable expression vector capable, in a eukaryotic transformant host cell, of expressing a DNA sequence of claim 23.
- 45 25. Eukaryotic host cells transformed with the vector of claim 24.
26. The cells of claim 25 that are yeast cells.
27. The cells of claim 25 that are mammalian cells.
- 50 28. The cell culture of claim 27 that is from a Chinese hamster ovary cell line.
29. A composition for treating a vascular disease or condition comprising a therapeutically effect amount of the plasminogen activator variant of any one of claims 1 to 22 in admixture with a pharmaceutically
55 acceptable carrier.



Patentansprüche

1. Fibrinolytisch wirksame Aminosäuresequenzvariante von menschlichem Gewebeplasminogenaktivator(t-PA), die N-gebundene oder O-gebundene Glykosylierung an einer Glykosylierungsstelle aufweist, die in nativem menschlichem t-PA nicht vorhanden ist, wobei die genannte Plasminogenaktivatorvariante eine erhöhte Halbwertszeit und verringerte Clearance rate bezogen auf nativen menschlichen t-PA aufweist.
2. Variante nach Anspruch 1, worin die genannte Glykosylierung eine N-gebundene Glykosylierung ist.
3. Variante nach Anspruch 2, worin eine N-gebundene Glykosylierungsstelle an einer Position der t-PA-Variante angefügt ist, die nach außen hin freiliegt.
4. Variante nach einem der Ansprüche 1 bis 3, die innerhalb ihrer Wachstumsfaktordomäne glykosyliert ist.
5. Variante nach Anspruch 1, die (1) ein Serin oder Threonin an Position 60 des nativen t-PA, (2) ein Asparagin an Position 64 und ein Serin oder Threonin an Position 66 des nativen t-PA, (3) ein Asparagin an Position 65 und ein Serin oder Threonin an Position 67 des nativen t-PA, (4) ein Asparagin an Position 67 des nativen t-PA, (5) ein Asparagin an Position 78 und ein Serin oder Threonin an Position 80 des nativen t-PA, (6) ein Asparagin an Position 79 und ein Serin oder Threonin an Position 81 des nativen t-PA, (7) ein Asparagin an Position 80 und ein Serin oder Threonin an Position 82 des nativen t-PA oder (8) eine Kombination aus beliebigen zwei oder mehreren der obigen (1) bis (7) enthält.
6. Variante nach Anspruch 5, worin die Variante ein Asparagin an Position 67 des nativen t-PA enthält.
7. Variante nach Anspruch 4, die durch das funktionelle Entfernen zumindest eines Abschnittes einer für Fibrinbindung verantwortlichen Domäne modifiziert ist, bei der aber eine mit jener von nativem t-PA vergleichbare Fibrinbindung wiederhergestellt ist.
8. Variante nach Anspruch 4, der zumindest ein Abschnitt der Fingerdomäne fehlt.
9. Variante nach Anspruch 8, die natürlichen t-PA umfaßt, dem die Aminosäuren 1-44 fehlen.
10. Variante nach einem der Ansprüche 1 bis 8, die an einer bekannten Glykosylierungsstelle von nativem menschlichem t-PA modifiziert ist.
11. Variante nach Anspruch 10, der funktionelle Kohlehydratstruktur an Aminosäure 184 fehlt.
12. Variante nach Anspruch 11, die bezogen auf nativen t-PA im Aminosäure-(1) 205 bis 215-Bereich, (2) 244 bis 255-Bereich, (3) 233-242-Bereich oder (4) zwei oder mehreren aus (1), (2) und (3) noch weiter modifiziert ist.
13. Variante nach Anspruch 4, die im Vergleich zu nativem t-PA gegen spezifische enzymatische Spaltung zur Zweikettenform resistent ist.
14. Variante nach Anspruch 13, die im wesentlichen frei von Zweikettenform ist.
15. Variante nach Anspruch 14, die in der Einzelkettenform durch stellengerichtete Mutagenese an einer Zweikettenspaltungsstelle stabilisiert ist.
16. Variante nach Anspruch 15, worin Position 275, numeriert entsprechend dem reifen Plasminogenaktivator, von einer anderen Aminosäure als Arginin eingenommen wird.
17. Variante nach Anspruch 16, bei der die genannte Aminosäure aus Glycin und Glutaminsäure ausgewählt ist.
18. Variante nach Anspruch 17, bei der die genannte Aminosäure Glutaminsäure ist.



19. Variante nach Anspruch 16, worin Position 277, numeriert entsprechend dem reifen Plasminogenaktivator, von einer anderen Aminosäure als Lysin eingenommen wird.

20. Variante nach Anspruch 19, bei der die genannte Aminosäure Isoleucin ist.

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21. Variante nach Anspruch 9, die gegenüber enzymatischer Spaltung an der Aminosäureposition 275 oder der 277-Stelle oder beiden resistent ist.

22. Variante nach Anspruch 21, ausgewählt aus:

10

des 1-44N67E275 t-PA,

des 1-44N67D184E275 t-PA,

des 1-44N67S184E275 t-PA,

des 1-44N67K213E275 t-PA,

des 1-44N67R210A211R212R213E275 t-PA,

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des 1-44N67R252E275 t-PA,

des 1-44N67K210E275 t-PA,

des 1-44N67E275I277 t-PA,

des 1-44N67D184E275I277 t-PA,

des 1-44N67S184E275I277 t-PA,

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des 1-44N67K213E275I277 t-PA,

des 1-44N67R210A211R212R213E275I277 t-PA,

des 1-44N67R252E275I277 t-PA, und

des 1-44N67K210E275I277 t-PA.

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23. DNA-Sequenz, die für die Variante nach einem der Ansprüche 1 bis 11 und 16 bis 22 kodiert.

24. Replizierbarer Expressionsvektor, der in einer eukaryoten Transformantwirtszelle zum Expressieren einer DNA-Sequenz nach Anspruch 23 fähig ist.

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25. Mit dem Vektor nach Anspruch 24 transformierte eukaryote Wirtszellen.

26. Zellen nach Anspruch 25, die Hefezellen sind.

27. Zellen nach Anspruch 25, die Säugetierzellen sind.

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28. Zellkultur nach Anspruch 27, die von einer chinesischen Hamster-Eierstock-Zelllinie stammt.

29. Zusammensetzung zur Behandlung einer Gefäßerkrankung oder -affektion, die eine therapeutisch wirksame Menge der Plasminogenaktivatorvariante nach einem der Ansprüche 1 bis 22 in Mischung mit einem pharmazeutisch verträglichen Träger umfaßt.

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Revendications

1. Variant de séquence d'acides-amino de l'activateur du plasminogène tissulaire (t-PA) humain doué d'activité fibrinolytique, qui possède une glycosylation par liaison à un atome d'azote ou par liaison à un atome d'oxygène à un site de glycosylation qui n'est pas présent dans le t-PA humain naturel, ledit variant d'activateur du plasminogène présentant une demi-vie accrue et une vitesse d'épuration réduite par rapport au t-PA humain naturel.

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2. Variant suivant la revendication 1, dans lequel la glycosylation est une glycosylation par liaison à un atome d'azote.

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3. Variant suivant la revendication 2, dans lequel un site de glycosylation par liaison à un atome d'azote est ajouté en une position du variant de t-PA qui est exposée à l'extérieur.

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4. Variant suivant l'une quelconque des revendications 1 à 3, qui est glycosylé à l'intérieur de son domaine de facteur de croissance.



5. Variant suivant la revendication 1, qui contient (1) une sérine ou thréonine en position 60 du t-PA naturel, (2) une asparagine en position 64 et une sérine ou thréonine en position 66 du t-PA naturel, (3) une asparagine en position 65 et une sérine ou thréonine en position 67 du t-PA naturel, (4) une asparagine en position 67 du t-PA naturel, (5) une asparagine en position 78 et une sérine ou thréonine en position 80 du t-PA naturel, (6) une asparagine en position 79 et une sérine ou thréonine en position 81 du t-PA naturel, (7) une asparagine en position 80 et une sérine ou thréonine en position 82 du t-PA naturel, ou (8) une association de deux ou plus de deux quelconques de (1) à (7) ci-dessus.
6. Variant suivant la revendication 5, qui contient une asparagine en position 67 du t-PA naturel.
7. Variant suivant la revendication 4, qui est modifié par élimination fonctionnelle d'au moins une portion d'un domaine responsable de la liaison à la fibrine, mais qui possède une liaison à la fibrine rétablie, comparable à celle du t-PA naturel.
8. Variant suivant la revendication 4, qui est dépourvu d'au moins une portion du domaine digitiforme.
9. Variant suivant la revendication 8, qui consiste en un t-PA naturel dépourvu des amino-acides 1 à 44.
10. Variant suivant l'une quelconque des revendications 1 à 8, modifié au niveau d'un site de glycosylation connu du t-PA humain naturel.
11. Variant suivant la revendication 10, dépourvu de structure glucidique fonctionnelle au niveau de l'acide-amino 184.
12. Variant suivant la revendication 11, présentant en outre des modifications supplémentaires, par rapport au t-PA naturel, (1) dans la région des amino-acides 205 à 215, (2) dans la région des amino-acides 244 à 255, (3) dans la région des amino-acides 233 à 242, ou (4) dans deux ou plus de deux des régions (1), (2) et (3).
13. Variant suivant la revendication 4, qui, comparativement au t-PA naturel, est résistant à un clivage enzymatique spécifique en une forme bicaténaire.
14. Variant suivant la revendication 13, pratiquement dépourvu de forme bicaténaire.
15. Variant suivant la revendication 14, qui est stabilisé sous forme monocaténaire par mutagenèse dirigée au niveau d'un site de clivage bicaténaire.
16. Variant suivant la revendication 15, dans lequel la position 275 numérotée en fonction de l'activateur du plasminogène mature est occupée par un amino-acide autre que l'arginine.
17. Variant suivant la revendication 16, dans lequel l'acide-amino est choisi entre la glycine et l'acide glutamique.
18. Variant suivant la revendication 17, dans lequel l'acide-amino est l'acide glutamique.
19. Variant suivant la revendication 16, dans lequel la position 277 numérotée en fonction de l'activateur du plasminogène mature est occupée par un amino-acide autre que la lysine.
20. Variant suivant la revendication 19, dans lequel l'acide-amino est l'isoleucine.
21. Variant suivant la revendication 9, qui est résistant à un clivage enzymatique au niveau de la position d'acide-amino 275 ou du site 277, ou bien au niveau de cette position et de ce site.
22. Variant suivant la revendication 21, choisi entre
 - le des 1-44N67E275 t-PA,
 - le des 1-44N67D184E275 t-PA,
 - le des 1-44N67S184E275 t-PA,
 - le des 1-44N67K213E275 t-PA,



le des 1-44N67R210A211R212R213E275 t-PA,
le des 1-44N67R252E275 t-PA,
le des 1-44N67K210E275 t-PA,
le des 1-44N67E275I277 t-PA,
5 le des 1-44N67D184E275I277 t-PA,
le des 1-44N67S184E275I277 t-PA,
le des 1-44N67K213E275I277 t-PA,
le des 1-44N67R210A211R212R213E275I277 t-PA,
le des 1-44N67R252E275I277 t-PA, et
10 le des 1-44N67K210E275I277 t-PA.

23. Séquence d'ADN codant pour le variant suivant l'une quelconque des revendications 1 à 11 et 16 à 22.

15 24. Vecteur d'expression répliquable capable, dans une cellule hôte transformante eucaryotique, d'exprimer une séquence d'ADN suivant la revendication 23.

25. Cellules hôtes eucaryotiques transformées avec le vecteur suivant la revendication 24.

20 26. Cellules suivant la revendication 25, qui sont des cellules de levure.

27. Cellules suivant la revendication 25, qui sont des cellules de mammifère.

25 28. Culture de cellules suivant la revendication 27, qui provient d'une lignée de cellules d'ovaire d'hamster chinois.

30 29. Composition pour le traitement d'une maladie ou affection vasculaire, comprenant une quantité à effet thérapeutique du variant d'activateur du plasminogène suivant l'une quelconque des revendications 1 à 22 en mélange avec un support pharmaceutiquement acceptable.

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