EXHIBIT A

X102

Characterization Studies of Human Tissue-type Plasminogen Activator Produced by Recombinant DNA Technology

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A crucial aspect of hemostasis in mammals is the control of the formation and dissolution of a fibrin matrix. The enzymatic system in plasma for fibrin formation consists of more than a dozen proteases and cofactors that act in series (Jackson and Nemerson 1980). Localization of these reactions is achieved by the requirement for the phospholipid surface, which is supplied by activated platelets at the site of trauma. Regulation of fibrin formation is achieved through control of protease activation, the requirement for a phospholipid surface at certain steps of the cascade, and feedback activation and inactivation reactions involving coagulation factors, other plasma proteins, and cell-surface proteins. The resulting fibrin matrix serves to prevent unwanted blood loss at the site of an injury.

Equal in importance to fibrin formation, fibrin dissolution is accomplished by a much simpler series of enzymatic reactions. The removal of fibrin is accomplished through the proteolytic cleavage of the insoluble fibrin strands into soluble fragments by the action of the serine protease plasmin (for a recent review, see Danø et al. 1985). Control of fibrin dissolution is achieved by the extent and localization of the activation of the glycoprotein plasminogen, the circulating zymogen form of plasmin. The best-known mammalian plasminogen activators are urokinase and tissue plasminogen activator (t-PA) (Danø et al. 1985). Through selective release and subsequent localization of urokinase, t-PA, and plasmin(ogen), selective and localized dissolution of fibrin is accomplished.

Although the body can effectively remove fibrin in the process of hemostasis, a number of human disease states result from the presence of unwanted blood clots. The desirability of a means to enhance the endogenous rate of clot lysis has long been recognized as essential to the effective treatment of such disease states as myocardial infarction, pulmonary embolism, deep vein thrombosis, and stroke (Verstraete 1980). This has led to the development of therapeutics (urokinase and streptokinase) that systemically activate plasminogen in the hope that sufficient plasmin will adsorb to the clot to enhance the rate of clot lysis. Although streptokinase and urokinase have both been used for such thrombolytic therapy, their usefulness in the management of thromboembolic disease remains to be established (Brogden et al. 1973; Paoletti and Sherry 1977). Unfortunately, plasmin is a relatively nonspecific protein (Robbins 1978; Castellino and Powell 1981; Robbins et al. 1981) that, as it circulates, will degrade fibrinogen as well as inactivate the coagulation factors V and VIII. This unwanted systemic proteolysis can lead to a serious bleeding state that can be as serious as the complication necessitating treatment.

For these reasons, a more effective and safe fibrinolytic agent is required. The ideal activator of plasminogen would selectively activate plasminogen in the vicinity of a clot while effecting minimal systemic activation. t-PA possesses these properties. This plasminogen activator specifically binds fibrin (Thorsen et al. 1972; Rijken and Collen 1981), and its ability to activate plasminogen is markedly increased in the presence of fibrin (Danø et al. 1985). These two properties (fibrin binding and activity enhancement) would be expected to give t-PA the specificity required of an effective and specific fibrinolytic agent. Increasing the level of t-PA in plasma was therefore expected to accelerate clot lysis while having minimal effect on circulating plasminogen. Although preliminary studies on t-PA produced by a melanoma cell line were promising (Weimar et al. 1981; Van de Werf et al. 1984), it was not clear whether sufficient material could be produced to make a sufficient fibrinolytic product at a cost-effective price using the natural sources (Collen et al. 1982). The application of recombinant DNA techniques to solve this problem led initially to the expression of the protein in bacteria (Pennica et al. 1983). We present here a report on subsequent work directed toward the production and characterization of recombinant tissue plasminogen activator (rt-PA) expressed in Chinese hamster ovary (CHO) cells, and we describe some of the physical properties of the resulting protein.

EXPERIMENTAL PROCEDURES

Protein. Recombinant t-PA (Activase®) is produced by Genentech, Inc. (South San Francisco, California). The protein was purified from the supernatants of CHO cells that have been transfected with a t-PA expression plasmid (Goeddel et al. 1983).

Amino acid sequence analysis. The protein samples were analyzed on the Beckman 890C protein se-

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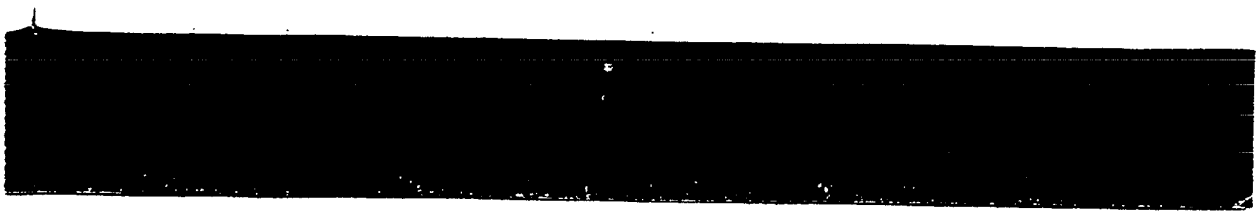


quencer for 11 cycles using 0.1 M Quadrol. The first cycle was performed without the addition of the coupling agent phenylisothiocyanate (PITC) to remove free amino acids that may coelute with the protein sample during the purification process. The next ten cycles were run with the addition of PITC and analyzed for PTH amino acids using a Waters M6000 high-performance liquid chromatography (HPLC) system with a 5-μm C_4-reversed-phase Microsorb column as described by Rodriquez et al. (1984). The amino acids were identified by comparison of retention times to a standard mixture of PTH amino acids.

Electrophoresis. SDS-PAGE was performed on 10% polyacrylamide gels according to the procedure of Laemmli (1970). Protein was visualized by staining with Coomassie blue R-250.

Plasmin activation of t-PA. rt-PA was converted to the two-chain form with human plasmin (Helena Laboratories). Plasmin (50 casein units) was covalently coupled to cyanogen bromide preactivated Sepharose (Pharmacia). rt-PA was applied to the immobilized plasmin column (5 ml) at a flow rate of 10 ml/hr. Conversion of rt-PA to the two-chain form was greater than 95% as determined by SDS-gel electrophoresis.

Separation of kringle and protease domains. Plasmin-treated rt-PA was reduced and carboxymethylated according to the procedure of Crestfield et al. (1963). The reduced, carboxymethylated two-chain t-PA (3 mg/2 ml) was applied to a Sephadex G-75 superfine column (1.5 × 100 cm) equilibrated in 0.1 M ammonium bicarbonate at pH 8.3. The column was developed under gravity flow, and the effluent was monitored for absorbance at 280 nm.

Tryptic digestion. Recombinant t-PA was reduced and carboxymethylated (RCM) according to the method of Crestfield et al. (1963). The product was dissolved in 0.1 M ammonium bicarbonate at 25°C with addition of L-1-*p*-tosylamino-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Cooper Biomedical) at an enzyme-to-substrate ratio of 1:100 (w/w) followed by a second addition after 8 hours. The digestions were stopped after 24 hours by freezing at −20°C.

HPLC tryptic mapping. The trifluoroacetic acid (TFA)-based tryptic map of rt-PA was achieved on a 5-μm Nova Pak-C18 column. Mobile phase A consisted of 0.1% TFA, and mobile phase B consisted of acetonitrile with 0.08% TFA. The linear gradient was carried out at a rate of 0.5% per minute for 25 minutes, followed by 1.0% per minute for 35 minutes. The sodium-phosphate-based tryptic map of rt-PA was performed under the same conditions except that 0.05 M sodium phosphate was the ionic modifier. The samples were loaded in 0.1 M ammonium bicarbonate and monitored at 214 and 280 nm. The separation was achieved on a Waters gradient liquid chromatograph that included two 610 pumps, a 720 controller, and a WISP injector.

Sialic acid determination. Sialic acid content was determined by the thiobarbituric acid (TBA) procedure of Warren (1959).

Glycosyl residue composition. Neutral and amino sugars were identified and quantitated by gas-liquid chromatography after hydrolysis in 4 N TFA (Nesser and Schweizer 1984) and conversion to alditol acetates (Albersheim et al. 1967).

Glycosyl-linkage analysis. Glycosyl-linkage composition was determined by methylation analysis (Lindberg 1972) as modified by Waeghe et al. (1983). Methylation was carried out with methyl iodide and methylsulfinylcarbanion in dimethyl sulfoxide. Methylated oligosaccharides were recovered by chromatography on C-18 Sep-Pak cartridges (Waters, Inc.) and then hydrolyzed, reduced, and acetylated. The resulting partially methylated alditol acetates were resolved and identified by combined gas-liquid chromatography/mass spectrometry.

RESULTS AND DISCUSSION

Structure of t-PA

The identification of cDNA clones that included the coding region of human t-PA resulted in the first complete picture of the primary structure of the protein (Pennica et al. 1983). The cDNA revealed a leader sequence of 35 amino acids preceding the amino-terminal serine residue of the secreted protein. The first 20–23 amino acids of the leader sequence are hydrophobic, typical of a signal sequence. This is followed by 12–15 hydrophilic amino acids of unknown function that do not appear on the secreted form of the protein. The function, if any, of this pre-pro-leader structure is unknown at present. Following processing of the pre-pro t-PA, the secreted form of the protein can be obtained as a single polypeptide chain containing 527 amino acids, 35 cysteine residues, and 4 potential N-linked glycosylation sites (Pennica et al. 1983; Pohl et al. 1984; Vehar et al. 1984). The protein sequences revealed by clones obtained by several independent laboratories are in agreement on the amino acid sequence of the protein, although some nucleic acid sequence differences have been noted (Ny et al. 1984; Fisher et al. 1985; Kaufman et al. 1985). Only one gene has been detected in the human genome (Pennica et al. 1983; Ny et al. 1984).

On the basis of sequence homology with other proteins, t-PA is believed to be a multidomain protein (Fig. 1). Starting from the amino-terminal end, it has been proposed to contain a potential type I finger domain (Banyai et al. 1983), a growth-factor domain (Banyai et al. 1983), two kringle domains (Pennica et al. 1983), and a serine protease or catalytic domain (Pennica et al. 1983).

A proteolytic cleavage site at Arg-275 (Pennica et al. 1983) divides the protein into two approximately equally sized polypeptide chains. The chain arising

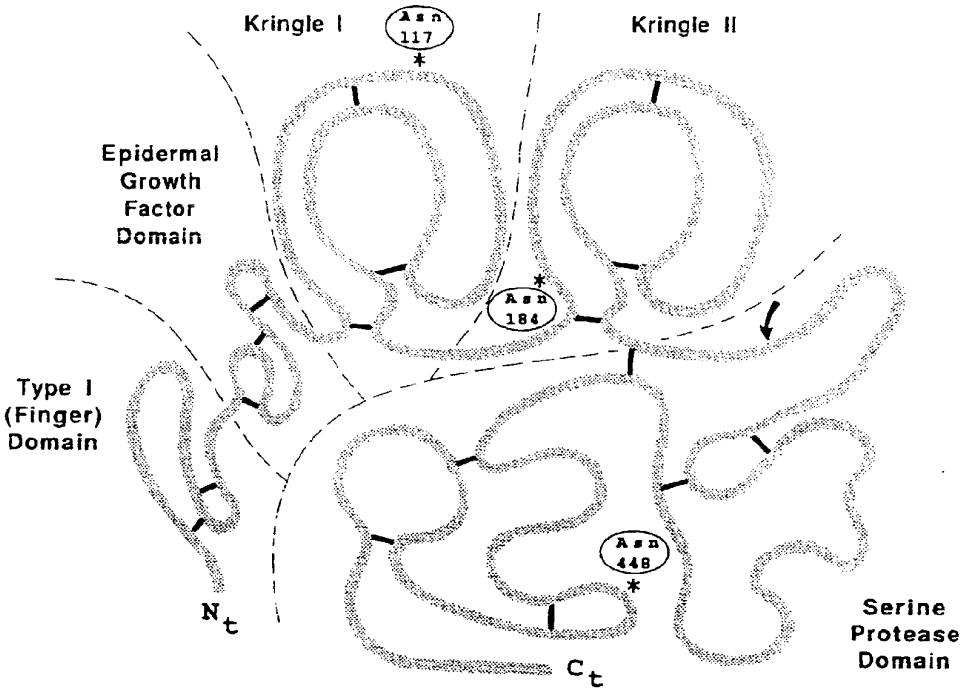


Figure 1. Schematic diagram of the domain structure of t-PA based on the domain proposals of Pennica et al. (1983) and Banyai et al. (1983). Stippled line represents the polypeptide chain. Solid bars represent the disulfide bonds. Sites of glycosylation are identified by asterisks, with the glycosylated asparagine residue numbers identified in the circle. Arrow indicates the position of the cleavage site at Asn-275. The amino(N_t)- and carboxy(C_t)-terminal ends are labeled. Dashed lines delineate the borders of the different domains.

from the carboxy-terminal portion of t-PA is the serine protease domain and shares considerable amino acid sequence homology with other members of this family of proteins (Strassburger et al. 1983). By analogy with other serine proteases, five intradomain disulfide bonds have been proposed for 10 of the 11 cysteine residues of this region of t-PA (Pennica et al. 1983). The extra cysteine residue of the protease domain has been proposed to connect the protease to the polypeptide chain derived from the amino terminus of the protein (Pennica et al. 1983). In this manner, the catalytic domain of t-PA remains covalently attached to the remaining domains following the proteolytic cleavage at position 275.

The amino-terminal 275 amino acids of t-PA contain the several proposed domains. The first 43 amino acids share limited sequence homology with type I finger structures (containing two disulfide bonds) (Banyai et al. 1983) that were originally identified in fibronectin (Peterson et al. 1983). These structures occur in a fragment of fibronectin known to be responsible for the fibrin affinity of the protein (Sekiguchi et al. 1981; Seidl and Hoermann 1983). By analogy with fibronectin, it was originally proposed that the type I structure was involved with the affinity of t-PA for fibrin (Banyai et al. 1983). However, it has subsequently been reported that expression of a form of t-PA lacking the finger domain results in a protein that will still bind fibrin (Kagitani et al. 1985). Further work will be required to clarify the function of this structure in t-PA.

Six amino acids separate the type I structure from a 34-amino-acid, three-disulfide-bond-containing region that shares sequence homology with growth factors (Banyai et al. 1983). Analogous growth-factor-like domains have been proposed to occur in factor X, factor IX, factor XII, protein C, and urokinase (Banyai et al. 1983; McMullen and Fujikawa 1985). The vitamin-K-dependent proteases (factor IX, factor X, protein C) each contains two growth-factor domains, of which one domain contains a β-hydroxyaspartic acid residue (Fernlund and Stenflo 1983; McMullen et al. 1983). An aspartic acid does not occur in the corresponding position of t-PA, such that this growth-factor domain would not be expected to contain this posttranscriptional modification. The function, if any, of the growth-factor domains in these proteases is unknown.

The growth-factor domain is separated by seven amino acids from the first of two proposed kringle structures (Pennica et al. 1983). A six-amino-acid segment separates the two t-PA kringle structures. Krin-

gles were first identified in prothrombin (Magnusson et al. 1975) and have subsequently been found in plasminogen (Claeys et al. 1976), urokinase (Günzler et al. 1982), and factor XII (McMullen and Fujikawa 1985). The first of the five plasminogen kringle structures has been shown to be responsible for the interaction of this protein with fibrin (Lerch et al. 1980; Thorsen et al. 1981). It is not known at present whether the t-PA kringles play a comparable role in interactions with fibrin.

Two amino acids follow the second kringle domain before the cysteine involved in the disulfide link to the protease is positioned (Pennica et al. 1983). The eleventh amino acid following this cysteine is Arg-275, which immediately precedes the serine protease domain. t-PA is structured such that very short polypeptide segments separate the various domains. This gives the protein considerable resistance to proteolysis in the native state, possibly reflecting the fact that the protein must function in the presence of the relatively nonspecific protease plasmin.

Host Selection

Escherichia coli was the expression system of choice for the early recombinant products (e.g., somatostatin, insulin, and the interferons). A number of factors were involved with this selection, including high expression levels, ease of growth, well-known genetics, suitable plasmids, and efficient promoters. The successful production of such products as human growth hormone eased concerns over the possibility of improper disulfide formation due to the intracellular reducing environment of *E. coli*. The production of fully active γ-interferon demonstrated that the lack of a glycosylation system in *E. coli* would not necessarily affect the product quality. *E. coli* was therefore the host of choice for expression of t-PA. Successful expression was achieved as evidenced by the ability of *E. coli* extracts to cause the dissolution of fibrin matrix (Pennica et al. 1983). More efficient expression was, however, achieved through the application of mammalian cell expression systems, wherein a properly processed and glycosylated molecule could be obtained from the cell-conditioned media (Collen et al. 1984; Zamarron et al. 1984).

Recombinant pharmaceutical products derived from the mammalian cell lines present unique safety concerns (Petricciani 1985). These can be addressed by careful validation of both the master working cell bank (Lubiniecki and May 1984; Martin 1985; Lubiniecki 1986; Palladino et al. 1986) and the process used to purify the products (Jones and O'Connor 1983).

The purification process for the production of rt-PA results in a highly purified product as shown in Figure 2. Nonreduced, a closely spaced doublet is observed that exhibits an apparent molecular weight of approximately 50,000. Upon reduction, the majority of the protein migrates with an apparent molecular weight of approximately 63,000. In addition, a faint band is visible with an apparent molecular weight of 35,000, indicating that a small amount of material has been cleaved to the two-chain form. Amino acid sequence analysis of the material confirms that this cleavage has occurred at Arg-275.

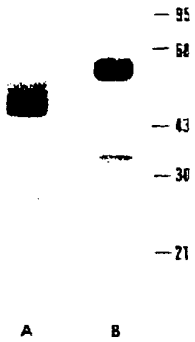
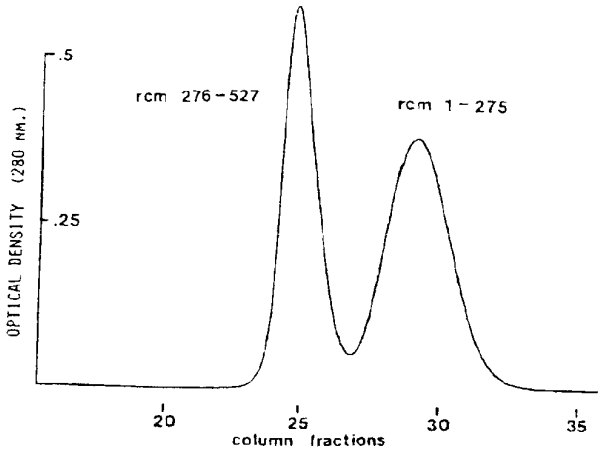


Figure 2. SDS-PAGE analysis of rt-PA. (*A*) Sample prepared without reduction; (*B*) sample reduced with mercaptoethanol prior to electrophoresis. The mobilities of molecular-weight standards are indicated at the right of the figure, with the molecular weight indicated (× 1000).

Plasmin-catalyzed Cleavage of t-PA

The large size of rt-PA complicates detailed analysis of the protein. Advantage was taken of the fact that limited proteolysis of rt-PA results in the generation of two polypeptide chains of approximately equal size. A single interdomain disulfide bond (Cys-264–Cys-395) has been proposed to link the kringle-containing domain (1–275) with the serine protease region (276–527). The action of plasmin on t-PA results in a single-peptide-bond cleavage that yields a two-chain, disulfide-bonded form of the protein (Wallen et al. 1983; Pohl et al. 1984). To confirm the site of plasmin cleavage within the t-PA molecule, the individual plasmin fragments of t-PA were prepared, isolated, and sequenced. rt-PA was fully converted to its two-chain form with immobilized plasmin and then dialyzed into 8 M urea, 0.5 M Tris-Cl at pH 8.3 as a modification of the procedure of Crestfield et al. (1963). Plasmin-treated t-PA was reduced with 10 mM dithiothreitol at 37°C for 30 minutes and carboxymethylated with 25 mM iodoacetate for 20 minutes at 25°C. The reduced and carboxymethylated kringle-containing region (RCM 1–275) and protease domain (RCM 276–527) were separated by gel filtration on a column of superfine Sephadex G-75 as seen in the elution profile (Fig. 3, top). RCM 276–527 and RCM 1–275 eluted in separate peaks as identified by SDS-gel electrophoresis of the collected fractions (Fig. 3, bottom). The chromatographic elution profile of this column was characterized by nonideal behavior of the separated components. RCM 276–527 and RCM 1–275



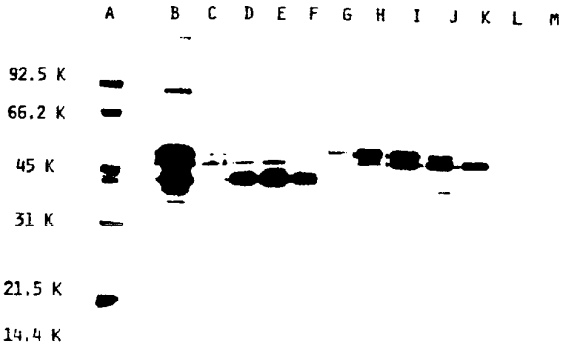


Figure 3. (*Top*) Gel filtration of RCM 1–275 and RCM 276–527. Elution profile of Sephadex G-75 superfine column (1.5 × 100 cm) in 0.1 M ammonium bicarbonate at pH 8.3. An aliquot (3 mg) of reduced and carboxymethylated t-PA (two-chain) was loaded onto the column. Fractions (5 ml) were monitored for optical density at 280 nm. The early and late eluting peaks were identified by amino acid sequencing and SDS-gel electrophoresis (Fig. 3) as protease (RCM 276–527) and kringle (RCM 1–275), respectively. (*Bottom*) Silver stain (Oakley) of G-75 profile. The elution profile of the Sephadex G-75 separation of two-chain RCM rt-PA (Fig. 2, top) was analyzed by SDS-PAGE. Clear separations of protease (lanes *D–F*) and kringle (lanes *H–K*) can be seen. Type I and type II kringles are also partially resolved by gel filtration (lanes *H* vs. *K*). (*A*) Molecular-weight standards; (*B*) RCM rt-PA, column load; (*C*) fraction 23; (*D*) fraction 24; (*E*) fraction 25; (*F*) fraction 26; (*G*) fraction 27; (*H*) fraction 28; (*I*) fraction 29; (*J*) fraction 30; (*K*) fraction 31; (*L*) fraction 32; (*M*) fraction 33.

were not expected to be resolved by gel filtration as they have similar molecular weights (28,600 and 31,900, respectively). Both components of the RCM mixture eluted with high molecular weights as determined by comparison with gel-filtration standards (data not shown). These separated fractions of rt-PA were useful for further characterization of this complex glycoprotein. Amino acid sequencing of RCM 1–275 and RCM 276–527 confirmed the first ten cycles for the amino terminus of rt-PA (serine 1) and for the site of plasmin cleavage after Arg-275 (Fig. 4, top and bottom). The presence of cysteine at position six of the amino terminus of the kringle-containing region was established by the yield of carboxymethyl cysteine. The purified fractions of rt-PA, RCM 1–275 and RCM 276–527, are well resolved and unequivocally identified by SDS-gel electrophoresis. The apparent size heterogeneity of the kringle-containing region (1–275, type I and type II) is clearly resolved by reduction and carboxymethylation of the sample prior to SDS-gel electrophoresis (Fig. 3, bottom). Further characterization of the structure and sequence of rt-PA was accomplished by examination of tryptic fragments.

Peptide Analysis of rt-PA

Reversed-phase HPLC has been demonstrated to be a powerful analytical technique suitable for the analysis of low-molecular-weight pharmaceuticals (U.S. Pharmacopeia 1985). It was hoped that this procedure would prove useful for the characterization of different production lots of rt-PA.

In 1978, it was shown that reversed-phase HPLC could be used to separate efficiently mixtures of peptides prepared by tryptic digestion of low-molecular-weight proteins (Hancock et al. 1978). Subsequently, this technique was extensively used for sequence determination of proteins, in which the tryptic peptides were isolated for characterization (Olieman and Voskamp 1984). In this application, the addition of TFA to the mobile phase as a volatile ionic modifier was particularly successful, as the purified peptides could be read-

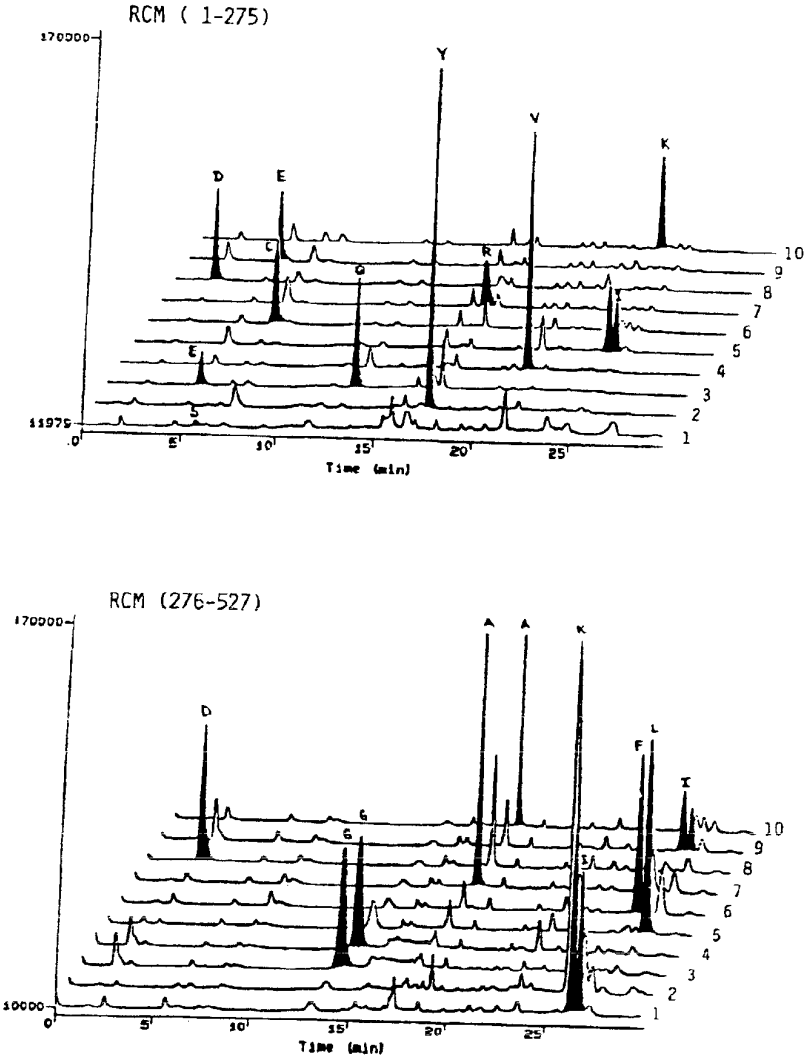


Figure 4. (*Top*) HPLC profiles obtained from ten cycles of automated amino acid sequencing RCM 1–275, i.e., kringle domain of rt-PA. (*Bottom*) HPLC profiles obtained from ten cycles of automated amino acid sequencing RCM 276–527, i.e., protease domain of rt-PA.

ily isolated in a salt-free form by lyophilization (Hearn and Hancock 1979; Olieman and Voskamp 1984). However, a number of studies have demonstrated that the addition of phosphate salts instead of TFA to the mobile phase gave improved peak shapes, better recoveries, and improved column lifetimes (Hancock and Harding 1984). The use of involatile phosphate severely hinders characterization of the purified peptides. Therefore, elution profiles of the reversed-phase tryptic map were performed for both the TFA and phosphate-containing mobile phases.

On the basis of the primary structure, tryptic digestion of rt-PA would be expected to yield 50 peptides. The elution profiles for the tryptic maps shown in Figures 5 and 6 demonstrate that reversed-phase HPLC can indeed resolve so complex a mixture. Approximately 44 and 51 peptides are resolved with the TFA and phosphate mobile phases, respectively. The higher number of peptides resolved in the phosphate-based system can be related to the increased resolving power of reversed-phase HPLC with this mobile phase due to mixed-mode chromatography, where the peptides in-

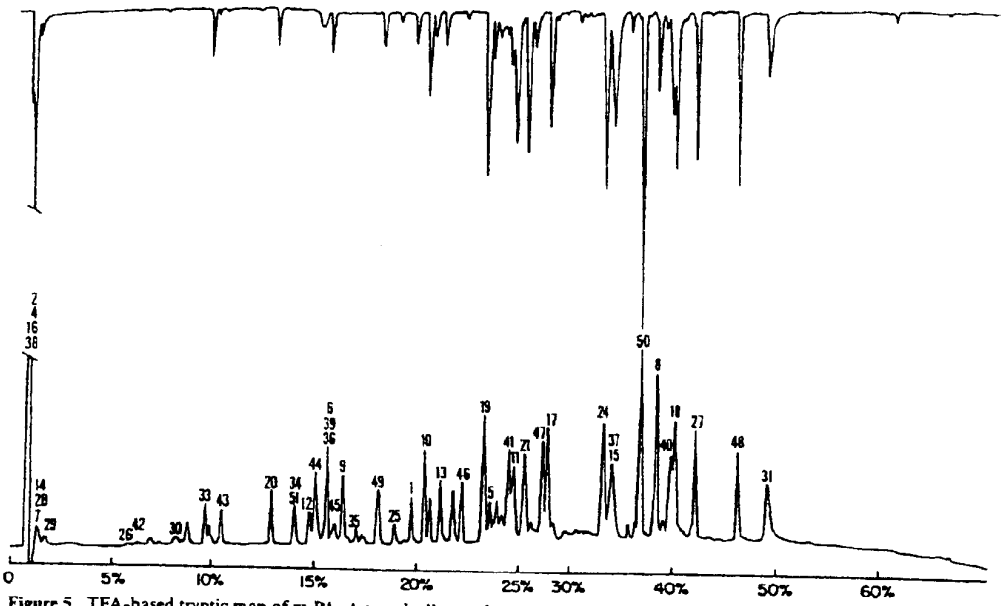


Figure 5. TFA-based tryptic map of rt-PA. A tryptic digest of rt-PA (200 μg in 0.2 ml) was applied to the column and analyzed as described in Experimental Procedures.

teract with the stationary phase by both polar and nonpolar interactions (Hancock and Sparrow 1981).

The presence of peptides in addition to the expected tryptic fragments can be related to heterogeneity in the protein caused by side reactions such as deamidation (particularly under the conditions of the tryptic digestion). Also, chymotryptic-like cleavages can result in additional peptide fragments. However, all peptide fragments were found to be consistent with the proposed structure for rt-PA. The average recovery of each peptide was approximately 50% of the 18 nmoles injected on the reversed-phase column.

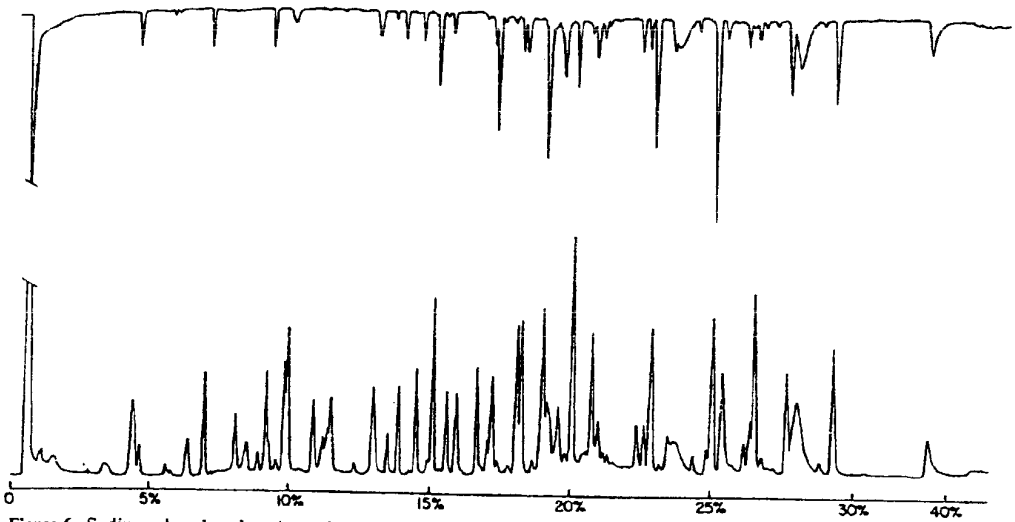


Figure 6. Sodium-phosphate-based tryptic map of rt-PA. A tryptic digest of rt-PA (70 μg in 0.1 ml) was applied to the column and analyzed as described in Experimental Procedures.

Figure 7 shows the primary structure proposed for rt-PA based on the cDNA sequence. Also shown is the location of the tryptic peptides reported by Pohl et al. (1984). All of the expected tryptic peptides were demonstrated to be present in the reversed-phase tryptic map. Each eluted peptide was identified by a combination of amino acid analysis and sequencing techniques. The identification of the tryptic peptides in the reversed-phase HPLC separation is shown in Figure 7. The order of elution follows approximately predicted retention times based on polarity measurements of the side-chain functional groups (Sasagawa and Teller 1984).

Carbohydrate Characterization of rt-PA

Oligosaccharide side chains of mammalian glycoproteins can be linked via the hydroxyl group of a serine or threonine residue (O-linked) or via the amide nitrogen of an asparagine residue (N-linked). O-linked oligosaccharides usually contain *N*-acetylgalactosamine and may also contain galactose and sialic acid. N-linked oligosaccharides are divided into two major categories: high-mannose oligosaccharides, composed of mannose and *N*-acetylglucosamine, and complex oligosaccharides, composed of mannose, *N*-acetylglucosamine, galactose, fucose, and sialic acid (Kornfeld and Kornfeld 1980).

The amino acid sequence of t-PA includes four potential N-linked glycosylation sites (Asn-X-Ser/Thr) (Marshall 1972): asparagine residues 117, 184, 218, and 448 (Pennica et al. 1983). Position 218, however, is not glycosylated in melanoma t-PA (Pohl et al. 1984; Vehar et al. 1984). The amino acid sequence at this site is Asn-Pro-Ser. The presence of proline in the middle position has been shown to interfere with glycosylation in other systems (Marshall 1974; Scheffer and Beintema 1974) and presumably is the cause for the absence of carbohydrate on Asn-218.

Melanoma-derived t-PA has been shown to exist in two variants (types I and II) that differ in carbohydrate content (Bennett 1983; Pohl et al. 1985). Melanoma-derived type I t-PA was found to contain N-linked glycans at positions 117, 184, and 448, whereas the type II t-PA lacks the glycan at position 184 (Bennett 1983; Pohl et al. 1985). The carbohydrate composition of melanoma-derived t-PA has been further investigated by Rijken et al. (1985). The protein was found to contain sialic acid, fucose, mannose, galactose, *N*-acetylglucosamine, and *N*-acetylgalactosamine. The type I and II forms were found to contain a high proportion of mannose, suggesting the presence of high-mannose

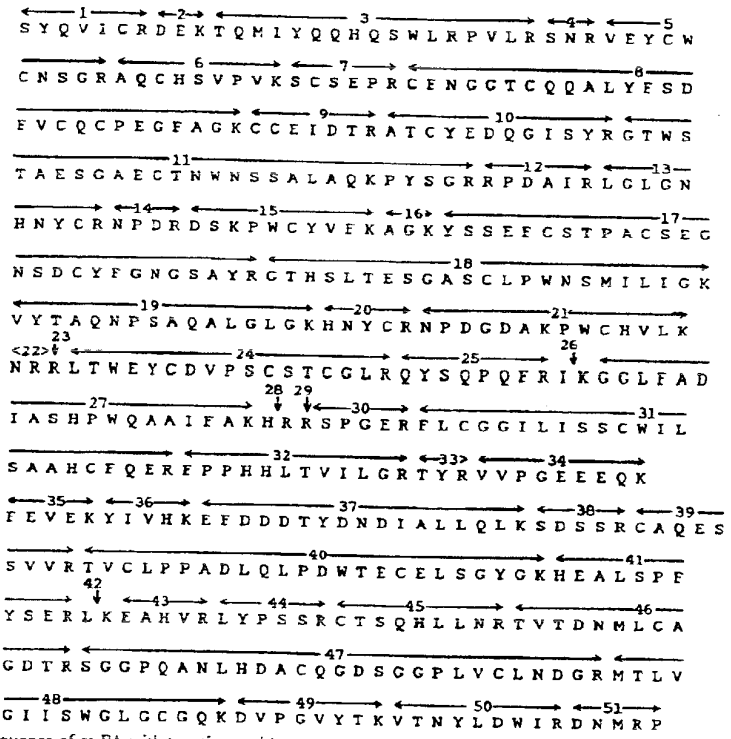


Figure 7. Linear sequence of rt-PA with tryptic peptides. Arrows define each peptide, and the number refers to the identity of the peptide listed in Fig. 4.

oligosaccharides. It has also been reported that both forms of melanoma-derived t-PA contain a high-mannose oligosaccharide at position 117 (Pohl et al. 1985).

The availability of glycosylated rt-PA allowed the characterization of the carbohydrate structures. The total carbohydrate content was determined using the thiobarbituric acid assay for sialic acid and the alditol acetate method for neutral and amino sugars (Table 1). The results indicate that rt-PA contains approximately 7% carbohydrate by weight. The presence of sialic acid, mannose, galactose, *N*-acetylglucosamine, and fucose was consistent with N-linked glycans of the fucosylated complex type, but the mannose content was higher than would be expected if the protein contained only complex oligosaccharides. The results suggest that rt-PA contains N-linked oligosaccharides of both the high-mannose and complex types.

The glycosyl linkage composition was determined by methylation analysis of pronase-digested rt-PA (Table 2). The linkage composition of rt-PA was consistent with a mixture of high-mannose and fucosylated complex oligosaccharides. The comparatively large amount of terminal mannose indicates the presence of high-mannose oligosaccharides. The linkage pattern observed with galactose indicates that sialic acid is attached to the 3-position of this residue in the complex oligosaccharides. Only a small amount of terminal galactose was observed, indicating that sialylation is nearly complete. Biantennary complex oligosaccharides contain 3,6-linked mannose as branching point; the presence of 2,4-linked mannose and 2,6-linked mannose indicates that rt-PA also contains some tri- and/or tetraantennary structures.

The attachment positions of the N-linked oligosaccharides were determined by characterizing tryptic glycopeptides. rt-PA was reduced, carboxymethylated, and digested with trypsin. Tryptic glycopeptides were isolated by lectin chromatography on concanavalin A–Sepharose followed by reversed-phase HPLC and were identified by amino acid analysis (Table 3). The major peptide containing residue 117 resulted from "chymotryptic" cleavage at tyrosine residue 126, whereas the peptides containing residues 184 and 448 resulted from the expected tryptic cleavage.

The neutral and amino sugar compositions of the isolated glycopeptides were determined by the alditol acetate method (Table 3). This analysis indicates that position 117 contains an oligosaccharide of the high-mannose type (probably $Man_5GlcNAc_2$ or $Man_6GlcNAc_2$). Positions 184 and 448 were found to contain fucosylated complex oligosaccharides.

In summary, rt-PA contains a high-mannose oligosaccharide at residue 117 and complex oligosaccharides at residues 184 and 448. This N-linked glycosylation pattern is similar to that reported for melanoma-derived t-PA (Pohl et al. 1985).

Table 1. Carbohydrate Composition of Intact rt-PA

Residue[a] (mole/mole rt-PA)				
Fuc	Man	Gal	GlcNAc	NeuAc[b]
2.5	9.8	3.7	7.4	2.7

[a] Abbreviations: Fuc, fucose; Man, mannose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; NeuAc, *N*-acetylneuraminic acid (sialic acid).

[b] Determined by thiobarbituric acid assay.

Table 2. Glycosyl Linkage Composition of rt-PA

Residue	Positions of O-methyl groups[a]	Deduced linkage	Area[b] (%)
Fuc	2,3,4	terminal	8
Man	2,3,4,6	terminal	13
Man	3,4,6	2-linked	11
Man	3,6	2,4-linked	2
Man	3,4	2,6-linked	trace
Man	2,4	3,6-linked	17
Gal	2,3,4,6	terminal	4
Gal	2,4,6	3-linked	16
GlcNAc	2,3,6	4-linked	27
GlcNAc	2,3	4,6-linked	3

[a] Determined from electron-impact mass spectra.

[b] From gas-liquid chromatography using flame-ionization detection.

CONCLUSIONS

The ability to safely increase the rate of lysis of unwanted fibrin clots in the vasculature has been a goal for many years. Current therapeutics have unwanted side effects that complicate safe and effective therapy. The efficacy of melanoma-cell-derived t-PA for such use was first demonstrated in two patients with venous thrombosis (Weimer et al. 1981). Subsequent studies of acute myocardial infarction documented t-PA-induced thrombolysis without significant fibrinogenolysis (Van de Werf et al. 1984). These studies indicated that t-PA had the properties of an ideal fibrinolytic agent. Unfortunately, the t-PA production level of these cell lines was sufficiently low that it was not clear whether sufficient material could be produced to treat effectively the more than one million potential patients that could benefit from the availability of a superior thrombolytic agent.

The generation of CHO cells capable of producing human t-PA has necessitated the development of large-scale tissue-culture fermentation and purification procedures (Builder and Grossbard 1986). The resulting product is highly purified and for the most part consists of the single chain form. The rt-PA has full catalytic and biologic activity (Collen et al. 1984; Zamarron et al. 1984). Analysis of the protein and its fragments has proven the fidelity of the CHO cells synthesizing a protein identical in amino acid sequence to the t-PA produced by human melanoma cells. The only differences due to expression in a nonhuman cell line would arise in the nature of the carbohydrate moieties. The availability of large amounts of rt-PA has allowed the preliminary analysis of these structures.

Table 3. Characterization of Tryptic Glycopeptides

Peptide[a]	Attachment residue no.	Molar ratio				Glycosylation type
		Fuc	Man	Gal	GlcNAc	
GTWSTAESGAECT-NWNSSALAQKPY	117	0.3	5.8	0.4	2.0	high mannose[b]
YSSEFCSTPACSE-GNSDCYFGNGSAYR	184	0.8	3.0	1.9	2.0	complex[c]
CTSQHLLNR	448	0.9	3.0	1.5	2.6	complex[c]

[a]Identification based on amino acid analysis.
[b]Normalized to 2 *N*-acetylglucosamine.
[c]Normalized to 3 mannose.

Human clinical trials have shown rt-PA (Activase®) to be an effective thrombolytic agent (Topol et al. 1985; Gold et al. 1986; Graor et al. 1986; Risius et al. 1986). Continued clinical testing will allow the evaluation of the potential benefit of rt-PA infusions in patients presenting with myocardial infarction, pulmonary embolism, deep vein thrombosis, and peripheral arterial occlusion. The success to date with rt-PA provides another example of the tremendous potential of recombinant DNA technology.

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