EXHIBIT N

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Influence of Carbohydrate Side Chains on Activity of Tissue-Type Plasminogen Activator (42336)

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Abstract. When messenger RNA (mRNA) from both untreated and phorbol ester-treated melanoma cells is translated in simple reticulocyte lysates, tissue-type plasminogen activator can be immunoprecipitated by an affinity-purified antibody as a ~52,000 mol wt protein, with no detectable biological (plasminogen activating) activity. When the reticulocyte lysate system is supplemented with a preparation of microsomal membranes, biological activity becomes detectable and a 63,000 mol wt protein can be immunoprecipitated with the same antibody. Furthermore, when natural tissue-type plasminogen activator (mol wt ≃ 70,000) is incubated with different glycosidases, distinct alterations in the electrophoretic mobility of the molecules are observed, together with alterations in the level of biological activity. While treatment with neuraminidase and β-galactosidase caused decreases in activity, α-mannosidase caused an increase. These results suggest that the carbohydrate part of the molecule can influence its biological behavior.

Plasminogen activators are serine proteases that convert plasminogen into plasmin and can thereby induce thrombolysis. Plasminogen activators occur in various organisms including bacteria. Those occurring in body fluids of animals and humans have been classified into serologically different urokinase-like plasminogen activators and tissue-type plasminogen activators. Tissue-type plasminogen activator was discovered in 1969 by Kok and Astrup in pig heart tissue (1). Human tissue-type plasminogen activator was first isolated from uterus tissue by Dingeman Rijken *et al.* (2). The human melanoma cell line, Bowes, was later found to be a more convenient source for the human product (3). By virtue of its clot-specific binding to fibrin, tissue-type plasminogen activator has distinct advantages over urokinase-like plasminogen activator as a thrombolytic agent for clinical use. Hence many efforts were made to develop production systems that are less time consuming than extraction from tissues or cell cultures. In particular, partial (4) and complete cDNA sequences (5, 6) as well as genomic clones (7) were isolated and sequenced. The cDNA sequences were furthermore introduced in high-yield procaryotic and eucaryotic expression systems (5, 6).

Natural tissue-type plasminogen activator is a one-chain glycoprotein with a mol wt of 72,000 (8). It can be cleaved by plasmin or trypsin into a disulfide-linked two-chain form (9). The COOH-terminal light chain possesses the serine protease active center (5). The NH_2-terminal heavy chain part exists in variants (10) and possesses a 43-residue-long amino-terminal region which is homologous with the fibrin-binding finger domain of fibronectin (11). A similar region cannot be found in urokinase-like plasminogen activator. Tissue-type plasminogen activator furthermore possesses two kringle structures found in certain other plasma proteins. The total mass of the carbohydrate moiety was estimated from the difference in electrophoretic mobility between the tissue-type plasminogen activator mRNA translation product obtained in a nonglycosylating cell-free system (12) (mol wt ~ 52,000) and that secreted by microinjected *Xenopus* oocytes (13) which was found to comigrate with the natural product (mol wt ~ 70,000).

The biological significance of the carbohydrate moiety has until now not been documented. Here we describe experiments which demonstrate that alterations in the carbohydrate can affect *in vitro* enzymatic activity of tissue-type plasminogen activator.

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Materials and Methods. *Cells and culture techniques.* The human melanoma cell line, Bowes, was originally obtained from Dr. D. B. Rifkin (Rockefeller University, New York, N.Y.). The cells were propagated in stoppered polystyrene flasks (175 cm^2, No. 3028, Falcon Plastics, Oxnard, Calif.) using Eagle's minimum essential medium, containing 10% v/v newborn calf serum, nonessential amino acids and 1.35 mg/ml of sodium bicarbonate. Before treatment with 12-*O*-tetradecanoylphorbol-13-acetate, the cultures were washed and 12-*O*-tetradecanoylphorbol-13-acetate was added to 20 ml of serum-free medium. Concentration (100 ng/ml) and incubation time (6 hr) were chosen after extensive kinetic studies (14).

Extraction of mRNA. Methods used for cell harvesting, extraction of RNA (phenol method using vanadyl complexes to inhibit ribonucleases), and purification of RNA (affinity chromatography on oligo(dT)-cellulose) are described in a previous paper from our laboratory (13).

Translation of mRNA in reticulocyte lysate and in oocytes. After washing the RNA pellets with 75% v/v cold ethanol, equal amounts of RNA from phorbol-treated and untreated cells were translated in rabbit reticulocyte lysates (N90, The Radiochemical Centre Ltd., Amersham, England) in the presence of L-[^{35}S]methionine as a label (12). For post-translational modification 1/10 vol dog pancreas microsomal membranes (a kind gift of Dr. J. Content, Pasteur Institute, Brussels, Belgium) was added to the reticulocyte lysates. The samples were used for electrophoretic analysis and tissue-type plasminogen activator assay. mRNA was also translated by microinjection in *Xenopus laevis* oocytes to yield secreted unlabeled biologically active tissue-type plasminogen activator protein and L-[^{35}S]-methionine-labeled product (13). The same RNA preparations were translated in both the absence and presence of 40 μg/ml of tunicamycin. To avoid RNA degradation, the tunicamycin was added only to the oocyte culture medium and not to the injected mRNA.

Reagents. 12-*O*-Tetradecanoylphorbol-13-acetate, tunicamycin, fibrinogen, α-mannosidase batches 85C-9510 and 61F-9655, β-galactosidase, and neuraminidase were purchased from Sigma Chemical Company (St Louis, Mo.). Homogeneous tissue-type plasminogen activator (t-PA) was prepared by concentration and extensive purification of Bowes melanoma cell supernatants (3, 14). Specific activities of all purified t-PA preparations exceeded 80,000 IU/mg protein. Electrophoretic purity of t-PA samples used in deglycosylation experiments was controlled by Coomassie brilliant blue staining and zymography (16), showing only a doublet protein band at 70,000 Da under nonreducing conditions. Electrophoretic separation of t-PA preparations under reducing conditions showed that different batches contained both one- and two-chain t-PA. With the use of homogeneous t-PA a polyclonal antibody was raised in rabbits. The IgG fraction of the rabbit serum was enriched in antibodies against t-PA by affinity chromatography on t-PA-coated Sepharose 6B. This affinospecific antibody neutralized all t-PA activities in fibrinolytic as well as amidolytic assays, whereas an Ig preparation of control rabbits not immunized with t-PA did not.

Enzymatic reactions on tissue-type plasminogen activator. Electrophoretically pure preparations of tissue-type plasminogen activator, containing 2060 urokinase units/ml, were prepared as previously described (3) and dissolved in 0.3 *M* NaCl, 0.01% v/v Tween 80. Equal amounts (200–300 μl, 35 μg protein/ml) were reacted with the enzymes α-mannosidase (5 IU, pH 4.5, 25°C), neuraminidase (1 IU, pH 5, 37°C), and β-galactosidase (0.5 IU, pH 4, 25°C) for different time intervals as indicated.

After reaction the samples were assayed for t-PA activity and analyzed by electrophoretic separation with Coomassie brilliant blue staining. Gels were run under reducing or nonreducing conditions as stated.

t-PA assay methods. Details of the assays for tissue-type plasminogen activator, immunoprecipitation techniques, and sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis are described in an earlier paper (13). Enzymatic activity of tissue-type plasminogen activator was assayed by measuring fibrinolysis in microtiter plates (15). For immunoprecipitation reactions the affinospecific antibody against t-PA was used. For SDS–polyacrylamide gel electrophoresis analysis of glycosidase-treated tissue-type plasminogen

activator samples, aliquots containing approximately 3.0 μg were desalted by Sephadex G-50 chromatography.

In some instances enzymatic activity of tissue-type plasminogen activator was evaluated by radial agar zymography, using the method described by Granelli-Piperno *et al.* (16).

Amidolytic assay of t-PA activity. Tissue-type plasminogen activator samples were treated with α-mannosidase and tested in an amidolytic assay using the chromogenic substrate S2288 (Kabivitrum, Stockholm, Sweden). The following solutions were used for the glycosidase experiments: (a) 50 μl α-mannosidase suspension in 3 *M* $(NH_4)_2SO_4$, 1 m*M* $(NH_4)OAc$, pH 7.5, was added to 250 μl t-PA solution (38 μg/ml in 0.3 *M* NaCl); (b) 50 μl 3 *M* $(NH_4)_2SO_4$ was mixed with 250 μl t-PA solution; (c) 50 μl α-mannosidase suspension was mixed with 250 μl of a 0.3 *M* NaCl solution.

The pH of all solutions was adjusted to 4.5 with 10^{-2} *N* HCl. After incubation at 25°C for different intervals (1, 2, 5, 8, 10, 15, 30, and 150 min), 50-μl samples were taken, diluted 20 times in ice-cold 100 m*M* Tris–HCl, pH 8.4 (25°C), 106 m*M* NaCl, and stored at −20°C until assay.

The amidolytic assay method for 1-ml cuvettes, as described by the manufacturer, was modified for microtiter plates (Sterilin, Middlesex, Great Britain). The wells were filled with either (i) 50 μl 100 m*M* Tris–HCl, pH 8.4 (25°C), 106 m*M* NaCl; (ii) 50 μl Tris–HCl buffer, 0.03% fibrinogen; or (3) 40 μl Tris–HCl buffer, 0.04% fibrinogen, and 10 μl thrombin solution (2×10^{-3} U/ml). The microtiter plate was stored in an incubator (37°C). After 2 hr, 50-μl samples of 20-fold diluted solutions (a), (b), and (c) and 50 μl of the S2288 substrate (5 m*M*) were added and mixed. Amidolytic activity was estimated from the release of *p*-nitroaniline, which was determined photometrically in a Titertek (Flow Laboratories, Irvine, Scotland). Absorbance (405 nm) increased linearly with time (5-min intervals), with a regression coefficient (r^2) of more than 0.998. Amidolytic activity was expressed as ΔA/min. Differences between duplicate determinations for a given sample were always less than 5%. Amidolytic activities of calculated t-PA quantities in the range of 10 ng could be determined. Fibrinogen preparations were free of detectable amidolytic activity. Thrombin added in the assay system to convert fibrinogen into fibrin had amidolytic activity (1×10^{-3} ΔA/min). All values obtained in the presence of fibrin were therefore corrected for this thrombin effect.

Results. *Effect of microsomal membranes on the translation of tissue-type plasminogen activator mRNA in the reticulocyte lysate system.* Confluent cultures of melanoma cells were washed and then incubated in serum-free medium containing 0 or 100 ng/ml of 12-*O*-tetradecanoylphorbol-13-acetate. After 6 hr the cells were collected for RNA extraction. The poly(A)-rich RNA was translated in the rabbit reticulocyte lysate system in the presence of L-[^{35}S]methionine as an intrinsic isotope. To some of the translational mixtures canine microsomal membranes were added for protein processing.

The translation products assayed for biological activity were examined by SDS–polyacrylamide gel electrophoresis. Only the samples obtained by addition of microsomal membranes contained detectable enzymatic activity: 0.050 and 0.340 IU/ml for the translation products of mRNA from uninduced and phorbol ester-induced cells, respectively. Furthermore, these activities could be neutralized by an antibody against t-PA (lower detection limit of the assay: 0.005 IU/ml). Autoradiographs of SDS–polyacrylamide gel electrophoresis analyses (Fig. 1) showed three prominent bands in the mol wt zone of ~50,000. Our previous studies (12) have allowed us to identify the upper (52,000) band (open diamonds) as being serologically related to tissue-type plasminogen activator. The middle band (50,000; filled triangle) is an artifact of the reticulocyte lysate system, being present on gels from translation mixtures to which no mRNA had been added. The lower band (48,000; open triangles) is from a protein that is serologically different from tissue-type plasminogen activator and that is translated from a phorbol-ester-induced mRNA. Addition of microsomal membranes resulted in appearance of faint 63,000 mol wt bands (arrowheads), with basically no alteration in the 52,000 mol wt bands. This 63,000 band has also been described in (5).

The autoradiograph shown in Fig. 2 is from a similar experiment, except that before SDS–

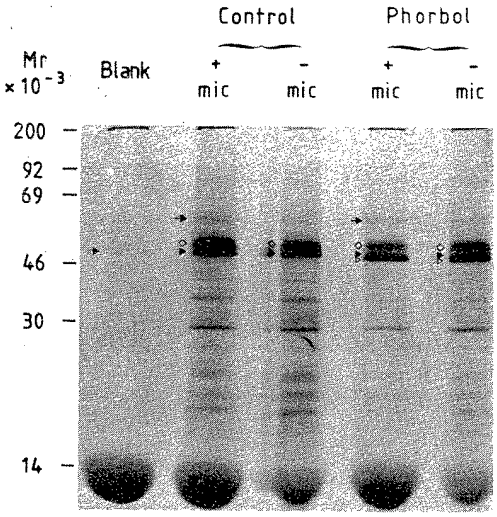

FIG. 1. Comparison by gel electrophoresis of microsomal membrane processed and unprocessed reticulocyte lysate translation products of poly(A)-rich RNA from melanoma cells. Equal amounts of poly(A)-rich RNA from untreated melanoma cells (control) and from cells treated for 6 hr with 100 ng/ml of 12-*O*-tetradecanoylphorbol-13-acetate (phorbol) were translated by reticulocyte lysates in the presence of L-[^{35}S]methionine as a label. Samples translated in the presence (+mic) or the absence (−mic) of canine pancreas microsomal membranes were applied to the gel slab without previous immunoprecipitation. Open diamonds indicate the 52,000 mol wt plasminogen activator band; filled triangles indicate the endogenous 50,000 mol wt band which is also seen in the blank reticulocyte lysate reaction; open triangles indicate the phorbol ester-induced 48,000 mol wt protein. Arrows indicate a 63,000 mol wt protein only seen after processing by microsomes.

polyacrylamide gel electrophoresis the translation products were immunoprecipitated with an affinity-purified antibody directed against tissue-type plasminogen activator. Again faint 63,000 mol wt bands were visible in the lanes corresponding to microsomal membrane-supplemented translation mixtures, providing proof that these bands are from activator-related proteins. Immunoprecipitation with an Ig preparation of a nonimmunized rabbit did not precipitate either a 52,000- or a 63,000-Da protein band from these translation mixtures. After addition of microsomal membranes the immunoprecipitable 52,000 mol wt band was decreased relative to the samples from translation without microsomal membranes. It cannot be said whether this was due to conversion of the 52,000 protein by glycosylation or to losses during the translation or immunoprecipitation procedure.

Effect of tunicamycin on the translation of tissue-type plasminogen activator mRNA in Xenopus oocytes. mRNA preparations such as those used for the previous experiments were translated by microinjection in *Xenopus* oocytes, and incubated in medium containing 0 or 40 μg/ml of tunicamycin, an inhibitor of glycosylation. Protein synthesis in this system was not significantly affected by tunicamycin: as compared to control oocytes, the tunicamycin-treated oocytes incorporated equal amounts of radioactivity into macromolecular protein. The media were harvested after 48 hr and titrated for plasminogen activator activity. Specificity was monitored by parallel assays in the presence of affinospecific antibody against tissue-type plasminogen activator. The results are shown in Table I. All enzymatic activities measured were neutralizable by specific antibodies, and fluids from mock-injected oocytes were inactive, indicating that the assay was

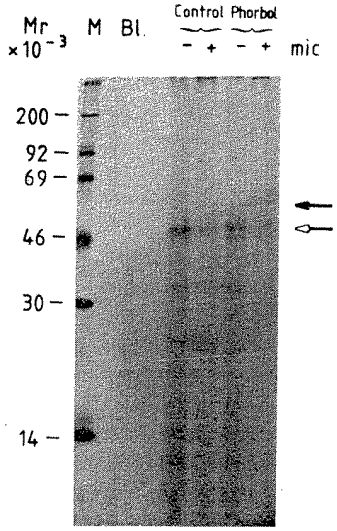

FIG. 2. Comparison by gel electrophoresis of microsomal membrane processed and unprocessed reticulocyte lysate translation products after immunoprecipitation. Same experiment as that illustrated by Fig. 1, except that before application to the gel, the translation products were precipitated by affinospecific antibody directed against tissue-type plasminogen activator. Open arrow indicates the unprocessed 52,000 mol wt band; filled arrow indicates the processed 63,000 mol wt band.

TABLE I. INHIBITORY EFFECT OF TUNICAMYCIN ON FORMATION OF BIOLOGICALLY ACTIVE TISSUE-TYPE PLASMINOGEN ACTIVATOR BY OOCYTES MICROINJECTED WITH TISSUE-TYPE PLASMINOGEN ACTIVATOR mRNA

mRNA injected	Total tissue-type plasminogen activator secreted (IU/ml)	
	Without tunicamycin in oocyte medium	With tunicamycin in oocyte medium
None	<0.005	<0.005
From control cells	0.023	0.013
From phorbol ester-treated cells	0.293	0.179

Note. Microinjected oocytes were incubated for 48 hr in the presence or absence of tunicamycin (40 μg/ml). Fluids were tested for tissue-type plasminogen activator by measuring fibrinolysis in microtiter plates. Biological activities were completely neutralizable by tissue-type plasminogen activator-specific antibody.

measuring translation of injected plasminogen activator mRNA. Also, as expected, levels of this mRNA were significantly higher in extracts from phorbol ester-treated than in untreated cells. With both kinds of mRNA tunicamycin caused a reduction in the enzymatic activity of the translation product. In a similar experiment the oocytes were incubated in medium containing [^{35}S]methionine and the secreted translation products were immunoprecipitated and analyzed by SDS–polyacrylamide gel electrophoresis. The autoradiograph shown in Fig. 3 clearly shows abolition by tunicamycin of the 72,000 mol wt product with appearance of a faint smear of immunoprecipitable proteins in the molecular weight zone of 52,000 to 70,000, suggesting that, in the presence of tunicamycin, the oocytes released nonglycosylated and partially glycosylated plasminogen activator peptides.

Effect of deglycosylation on enzymatic activity of tissue-type plasminogen activator. To test the effect of deglycosylation on the enzymatic activity of plasminogen activator, reaction mixtures were prepared containing 35 μg/ml (final concentration) of an electrophoretically pure tissue-type plasminogen activator preparation, together with either neuraminidase, β-galactosidase, or α-mannosidase. Reaction mixtures without added enzyme or with only the enzymes and no activator were also prepared. Further reaction conditions (temperature, pH, salt concentration) depended on the enzyme present and were as described under Materials and Methods. After 2 hr incubation and completion of the reaction, residual plasminogen activation was assayed by radial zymography or by fibrin plate assay. Alterations in molecular weight were studied by electrophoresis in slab gels.

The fibrin plate assay results, as summarized in Table II, indicate that neuraminidase and β-galactosidase reduced the biological activity of tissue-type plasminogen activator by 15 to 85%. In contrast, α-mannosidase caused a re-

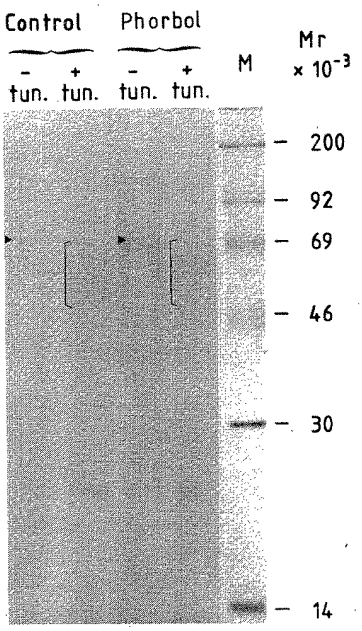

FIG. 3. Comparison by gel electrophoresis of *Xenopus* oocyte translation products of melanoma cell RNA. Effect of tunicamycin. Oocytes were injected with poly(A)-rich RNA from noninduced melanoma cells (control) and phorbol ester-treated cells (phorbol). After translation for 48 hr in the presence (+tun) or absence (−tun) of tunicamycin the L-[^{35}S]methionine-labeled products were immunoprecipitated from the oocyte culture fluids with an affinity-purified antibody against tissue-type plasminogen activator. The arrows indicate the 70,000-Da oocyte-secreted plasminogen activator. The brackets indicate the 52,000- to 70,000-Da smear of plasminogen activator molecules in different stages of glycosylation.

TABLE II. EFFECT OF PARTIAL DEGRADATION OF TISSUE-TYPE PLASMINOGEN ACTIVATOR BY GLYCOSIDASES ON ITS ENZYMATIC ACTIVITY

	Residual activity[a] after incubation					
	IU/ml					Average (% of tissue plasminogen activator alone)
Expt:	1	2	3	4	5	
Tissue-type plasminogen activator alone	270	80	220	1872	1852	100
Tissue-type plasminogen activator + neuraminidase	41	35	—	—	—	47
Tissue-type plasminogen activator + β-galactosidase	—	60	190	—	—	81
Tissue-type plasminogen activator + α-mannosidase	340	140	350	3040	2860	155
α-Mannosidase alone	—	—	—	≤5	0	NA

[a] Activity tested by measuring fibrinolysis in microtiter plates, using urokinase as a standard.

producible increase by about 55%. α-Mannosidase by itself did not score positive in the plasminogen activator assay, thereby eliminating the possibility that its enhancing effect on enzymatic activity was due to possible proteolytic contaminants of the preparation. The increased observed with α-mannosidase treatment was completely abolished by subsequent treatment with neuraminidase (data not shown). As shown by Fig. 4A the enhancement of tissue-type plasminogen activator by incubation with α-mannosidase could also be demonstrated by radial zymography. The change in molecular mass of tissue-type plasminogen activator by treatment with α-mannosidase is illustrated in Fig. 4B: the original 67,000/70,000 mol wt products are completely converted into a 63,000/66,000 doublet. Immunoblot analysis with the affinospecific antibody confirmed these observations (data not shown).

Amidolytic activities of t-PA, α-mannosidase, and combinations of both. Experiments using a chromogenic substrate assay were done to test amidolytic activity of t-PA, α-mannosidase, and combinations of both. These experiments were necessary to verify that the preparation of α-mannosidase, although being electrophoretically homogeneous, did not contain proteolytic activity which might be responsible for the enhancing effect of t-PA activity. Enzyme mixtures (t-PA, α-mannosidase, or t-PA plus α-mannosidase) were incubated at 25°C, pH 4.5, for 10, 30, or 150 min and then assayed for amidolytic activity, both in the absence and the presence of fibrinogen or fibrin (see Materials and Methods). The latter was done because it is known that the presence of fibrin influences the proteolytic activity of t-PA. The results of a representative experiment are tabulated in Table III. α-Mannosidase was devoid of any measurable amidolytic activity. In the absence of fibrinogen or fibrin, α-mannosidase did not affect the activity of t-PA, indicating that it did not convert t-PA from its one-chain to its two-chain form. In the presence of either fibrinogen or fibrin, the amidolytic activity of t-PA was consistently increased. A kinetic analysis of the enhancement of t-PA activity by α-mannosidase in the chromogenic substrate assay is shown in Fig. 5. The experimental design was similar to that of the experiment shown in Table III, except that shorter incubation times were chosen, allowing for extrapolation to zero time. The curves confirm that, in the presence of fibrin, α-mannosidase treated t-PA is more active than the native form and that, under the conditions used the conversion is near complete at 10 min reaction time.

Discussion. The molecular weight of melanoma cell-derived tissue-type plasminogen activator was originally estimated to be 72,000

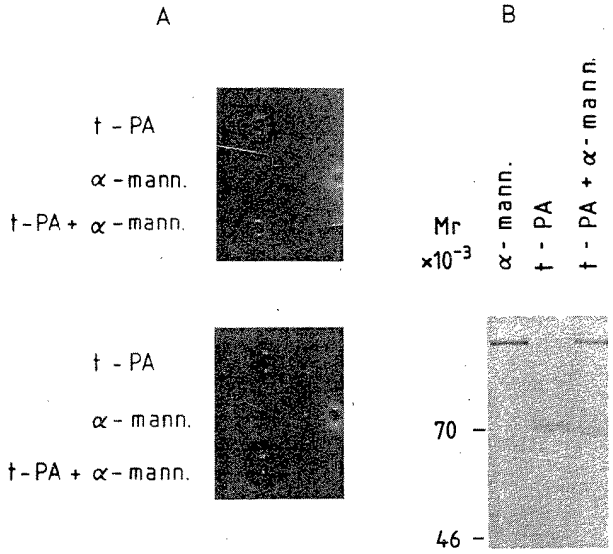

FIG. 4. Fibrin agar zymogram and gel electrophoretic analysis of native and α-mannosidase-treated tissue-type plasminogen activator. (A) Equal amounts of tissue-type plasminogen activator were incubated in the presence or absence of α-mannosidase. After reaction the tissue plasminogen activator samples and the glycosidase samples were tested for fibrinolytic activity by appearance of lysis zones on the indicator gel. After ~90 min the gels were photographed by darkfield illumination. t-PA = tissue-type plasminogen activator. (B) Samples of 3 μg tissue-type plasminogen activator were incubated for 5 hr with α-mannosidase. Activator as well as enzyme proteins were visualized by Coomassie brilliant blue staining.

(7). Subsequent studies (8) have confirmed this estimate or have yielded rather lower values, ranging down to a 63,000/65,000 doublet (5). Evidence that tissue-type plasminogen activator is glycosylated comes from its chromatographical behavior on lectin columns (7) but also from comparison of *in vitro* mRNA translation products. Thus, translation in rabbit reticulocyte lysates yielded an immunoprecipitable protein of mol wt 52,000. In contrast, translation of the same mRNA in oocytes yielded a secreted protein of mol wt 70,000 (12, 13). From the nucleotide sequence of the t-PA cDNA, a mol wt of 59,000 was predicted for the peptide part, i.e., less than the molecular weight of the natural product but more

TABLE III. AMIDOLYTIC ACTIVITY OF UNTREATED AND α-MANNOSIDASE-TREATED TISSUE-TYPE PLASMINOGEN ACTIVATOR IN THE ABSENCE AND PRESENCE OF FIBRINOGEN AND FIBRIN

Enzyme mixture[b] tested	Amidolytic activity[a] (ΔA/min/100 ng t-PA × 10^3) in the presence of								
	Nil			Fibrinogen[b]			Fibrin[b]		
	10[c]	30	150	10	30	150	10	30	150
t-PA	20.5	20.75	19.75	22.8	22.5	22.7	24.1	22.9	29.7
t-PA + α-mannosidase	19.4	20.5	20.9	26.4	26.3	24.2	31.0	28.8	34.6
α-Mannosidase alone		n.d.[d]			n.d.			n.d.	

[a] Chromogen release assay. (Standard errors of means always less than 2, $N = 3$.)
[b] See Materials and Methods.
[c] Incubation time (min) of enzyme mixtures before assay.
[d] Not detectable.

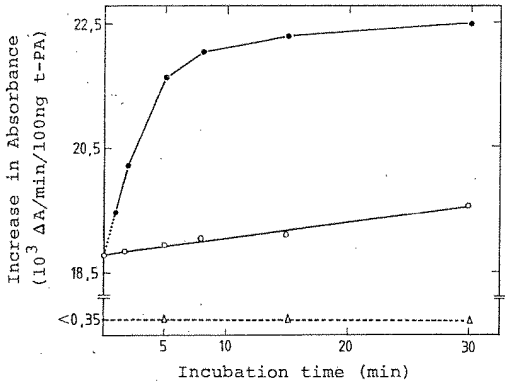

FIG. 5. Time kinetic analysis of the enhancement of tissue-type plasminogen activator amidolytic activity by α-mannosidase. Experimental design similar to that of Table III, column 3 (Fibrin). (○) Untreated t-PA; (●) t-PA treated with α-mannosidase for indicated time intervals; (△) α-mannosidase alone.

than the molecular weight of the unprocessed reticulocyte lysate translation product (17, 12).

The fact that the rabbit reticulocyte translation product has no demonstrable plasminogen activating potential, but the product of oocytes clearly possessed such activity (13) suggested that the carbohydrate moieties are important for biological activity. This is further supported by the evidence presented here.

Addition of microsomal membranes to the reticulocyte lysate system resulted in an increase in molecular weight of the immunoprecipitable product and acquisition of measurable biological activity. Conversely, addition of the glycosylation inhibitor, tunicamycin, to the oocyte translation system resulted in a decrease in both molecular mass and biological activity. Finally, treatment of the natural product with glycosidases resulted in a decrease in molecular weight and changes in biological activity, which depended on the specificity of the glycosidase used.

The *in vitro* translation in rabbit reticulocyte lysate supplemented with canine pancreas microsomal membranes is a complex system involving not only glycosylation but also signal peptide cleavage and possibly membrane-aided folding of complex molecules. The signal peptide of t-PA comprises an estimated 20–23 amino acids. Although the general assumption is that signal peptides do not play a role in determining biological activity, but are essential in directing proteins into the secretory pathway, we cannot exclude that, in the case of t-PA, cleavage of the signal peptide might enhance its biological activity. Translation experiments using SP6 RNA polymerase-generated transcripts from vector constructions, possessing or lacking the code for the signal peptide, might resolve this question.

The molecular weight of the oocyte-translated tissue-type plasminogen activator product (~70,000) is about equal to that of the natural cellular tissue-type plasminogen activator (~72,000). The reticulocyte lysate-translated tissue-type plasminogen activator has a mol wt of only 52,000. Addition of microsomal membranes to the reticulocyte lysate caused an increase in mol wt from ~52,000 to ~63,000, rather than the expected 70,000 to 72,000.

Thus, a difference of 7000 was noted between the molecular weights of the immunoprecipitable band (52,000) of the reticulocyte lysate translation (12) and the calculated 59,000 from the cDNA (17). Remarkably, a similar difference between the microsomal membrane-glycosylated 63,000 mol wt band from reticulocyte lysates and the oocyte-generated or natural t-PA (70,000) was observed. Perhaps this difference results from cleavage of a fragment of this size in the reticulocyte lysates. The 63,000 mol wt product, in contrast to the 52,000 mol wt unglycosylated protein, had detectable enzymatic activity, suggesting that glycosylation is important for enzymatic activity.

The same conclusion was reached from the alterations in enzymatic activity of plasminogen activator caused by treatment with glycosidases. Neuraminidase and β-galactosidase caused a decrease in biological activity accompanied by appearance of a second component of lower molecular weight. The decrease in total enzymatic activity may be due to decreased or even abolished activity of these partially deglycosylated forms. Treatment with α-mannosidase on the other hand caused complete conversion of the original to a slightly lighter form, accompanied by an increase in enzymatic activity.

On many electropherograms both the original and the partially deglycosylated forms of tissue-type plasminogen activator appeared as

doublets. In all likelihood these doublets are the equivalents of variants I and II (10).

A precedent for the effect of carbohydrate side chains in enzyme molecules on biological activity is the recent observation of Chavin and Weidner that neuraminidase can cause a decrease in both molecular mass and biological activity of clotting factor IX (18). Recent work (19) showed that endo-β-*N*-acetylglucosaminidase H-treated tissue-type plasminogen activator had unchanged fibrin-directed properties and effectively activated plasminogen. The study was, however, performed with a glycosidase differing from α-mannosidase and did not show alterations in electrophoretic mobility of the deglycosylated protein.

The carbohydrate moiety of various glycoproteins (e.g., cell membrane glycoproteins, lectins, . . .) has been shown to play an important role in ligand–receptor recognition. Accordingly, the carbohydrate part of tissue-type plasminogen activator may also be essential for substrate recognition. In the case of t-PA, it has been suggested that the affinity for fibrin could be due to the N-terminal region which is homologous to the finger domains of fibronectin (11). Unexpectedly, however, no glycosylation sites occur in this 43-amino acid-long region, while three and one such sites do occur, respectively, in the two kringle domains and in the C-terminal serine protease part of the molecule. Perhaps specific substrate affinity of t-PA results from cooperation between the fibronectin-homologous region and the glycosylated region(s) of the molecule.

This concept is supported by our observation that in chromogen release assays the enhancement of t-PA activity by α-mannosidase was only detectable when these assays were run in the presence of fibrin(ogen). It seems likely, therefore, that the α-mannosidase-sensitive glycosylation site is localized in the fibrin-binding kringle structures. Further support for this thesis is being sought by the sequencing of the carbohydrate side chains and their precise localization on the t-PA polypeptide.

Tissue-type plasminogen activator, produced in *Escherichia coli* by recombinant DNA techniques, has biological activity despite the fact that it is not glycosylated (5). This is not in conflict with our studies since they do not imply that the unglycosylated product is completely inactive, but only that it is less active than the natural product. A careful comparison of the specific activities (number of units per milligram protein) would be needed to confirm this view.

Carbohydrate groups of glycoproteins have also been shown to be important in tissue repartition and pharmacokinetics. Hence, another role of the carbohydrate part of t-PA may be essential for optimal distribution between body compartments and interaction with its inhibitors (20).

Our observation that treatment of t-PA with α-mannosidase causes an increase in plasminogen-activating activity goes along with a recent report (21) indicating that type I and type II t-PA, which differ by carbohydrate moiety, also differ in specific activity. Perhaps, the enhancing effect of α-mannosidase may be exploited to improve the therapeutic potential of tissue-type plasminogen activator. Thus, it may enable one to obtain undiminished therapeutic effects with lower doses than those needed with the intact product. Also, it might provide a conceptual basis for improving the pharmacokinetic properties of the product without diminishing its enzymatic potential. Tissue-type plasminogen activator produced by recombinant DNA technology may possibly be modified in its carbohydrate composition by using mannose-deficient systems or expression systems with other glycosylation patterns, e.g., yeast species.

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