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Effects of N-Glycosylation on in Vitro Activity of Bowes Melanoma and Human Colon Fibroblast Derived Tissue Plasminogen Activator

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ABSTRACT: Tissue-type plasminogen activator (t-PA), when isolated from human colon fibroblast (hcf) cells, is N-glycosylated differently than when isolated from the Bowes melanoma (m) cell line (Parekh et al., 1988). Both hcf- and m-t-PA can be separated into type I t-PA (with three occupied N-glycosylation sequons, at Asn-117, -184, and -448) and type II t-PA (with two occupied sequons, at Asn-117 and -448). Oligosaccharide analysis of each of these types of t-PA indicates that hcf-t-PA and m-t-PA have no glycoforms in common, despite having the same primary amino acid sequence. We have therefore compared in vitro the enzymatic activities and fibrin binding of type I and type II hcf- and m-t-PA with those of aglycosyl t-PA isolated from tunicamycin-treated cells. Plasminogen activation kinetics were determined by using an indirect amidolytic assay with Glu-plasminogen and a chromogenic plasmin substrate. In the absence of stimulator, there was little difference in activity between type I and type II t-PA, but the activity of aglycosyl t-PA was 2-4-fold higher than that of the corresponding glycosylated t-PA. In the presence of a fibrinogen fragment stimulator, the K_{cat} value of type II t-PA was approximately 5-fold that of type I t-PA from the same cell line, while the K_m values for activation of Glu-plasminogen were similar (0.13-0.18 μM). The stimulated activity of glycosyl t-PA was similar to that of type II t-PA. No effect of glycosylation was seen when each t-PA was assayed directly with H-D-Val-Gly-Arg-*p*-nitroanilide. In a clot lysis assay, type II t-PA was 23-26% more active than the type I t-PA from the same cell line, while type I and type II m-t-PA were about 30% more active, respectively, than type I and type II hcf-t-PA. The fibrin binding ability of each t-PA correlated very well with clot lysis activity but not with the stimulated indirect amidolytic activity. Together, these results suggest that sequon occupancy of Asn-184 (in addition to Asn-117 and -448) significantly decreases the fibrin-dependent stimulation of t-PA activity. Occupancy of sequons Asn-117 and -448 decreases the unstimulated activity of t-PA, and the nature of the oligosaccharide at Asn-448 influences both fibrin binding and clot lysis activity.

Human t-PA[1] is a glycoprotein containing four potential N-glycosylation sites (Pennica et al., 1983). In type I t-PA, only three of these are occupied, and in type II t-PA, only two are occupied (Pohl et al., 1984). We have recently defined in detail the N-glycosylation of t-PA derived from a human colon fibroblast (hcf) cell strain and a Bowes melanoma (m) cell line (Parekh et al., 1989). In both these cases it was shown that N-glycosylation served to create a number of variants (*glycoforms*) of the t-PA polypeptide and that there were no common glycoforms between the t-PA derived from the two cell lines, and hence the t-PA from these two cell lines are chemically distinct. We now assess some of the effects of these defined differences in N-glycosylation on the specific activity

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[1] Abbreviations: t-PA, tissue-type plasminogen activator; hcf, human colon fibroblast; m, Bowes melanoma; mAU, milli absorbance units; ELISA, enzyme-linked immunosorbent assay; SDS-PAGE, gel electrophoresis in the presence of sodium dodecyl sulfate; DMEM, Dulbecco's modified Eagle's medium.

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of the type I and type II forms of hcf- and m-t-PA, as measured by four different in vitro methods, and also the ability of each of these forms of t-PA to bind fibrin clots. The activity of aglycosyl t-PA isolated from both hcf and m cells cultured in the presence of tunicamycin is also examined. The results indicate significant differences in plasminogen activation not only between the type I, type II, and aglycosyl forms of a single t-PA polypeptide, but also between type I (and II) t-PA derived from hcf- or m-t-PA; i.e., both qualitative and quantitative differences in t-PA N-glycosylation influence its in vitro enzymatic activities.

Materials and Methods

Preparation of t-PA Samples. m-t-PA was obtained from American Diagnostica Inc. or isolated from mycoplasma-free clones of Bowes melanoma cells as described below. Two-chain hcf-t-PA and m-t-PA was used in all experiments and fractionated into type I and type II forms as described elsewhere (Parekh et al., 1989). The t-PA's were purified by immunoaffinity chromatography and gel filtration procedures. Preparations purified by different methods (e.g., Zn chelate Sepharose replacing ion-exchange chromatography, or employing *Erythrina caffra* inhibitor instead of anti-t-PA monoclonal antibody for affinity chromatography) all had similar specific activities in the stimulated indirect amidolytic assay. Aglycosyl t-PA was isolated from tunicamycin-treated cells as follows. Bowes melanoma (m) cells were obtained from D. B. Rifkin, New York University Medical School, and rendered free of mycoplasma by G. J. McGarrity, Department of Microbiology, Institute for Medical Research, Camden, NJ. Mycoplasma-free clones were screened for t-PA activity, and the highest producing clone was used for this work. The human colon fibroblast (hcf) cell strain CCD-18Co was obtained from the American Type Culture Collection, Rockville, MD (ATCC No. CRL 1459). Cells were grown to 90% confluency in T-150 culture flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with either 6% (m) or 10% (hcf) fetal bovine serum. Cells were incubated for 1.3 h with or without 1 μg/mL B2 tunicamycin (Boehringer-Mannheim) in serum-free medium (DMEM containing 0.5% lactalbumin hydrolysate, Sigma Chemical Co.), after which the cells were allowed to condition in fresh medium for 22 h in the presence or absence of tunicamycin. For each cell type, a set of control and tunicamycin-treated flasks received either 50 μCi/mL D-[2-^{3}H]mannose (18.3 Ci/μmol, Amersham) with decreased glucose (0.3 instead of 1 mg/mL) or 50 μCi/mL L-[4,5-^{3}H]leucine (161 Ci/μmol, Amersham) with decreased unlabeled leucine (21 instead of 105 μg/mL). These were subsequently referred to as preparations 1 and 2, respectively (see Results, Table III). Since aglycosyl t-PA may not be efficiently secreted (Little et al., 1984), t-PA was isolated from both conditioned medium and cells. Cells were extracted by agitating with 2 M KSCN for 30 min, and the extract was combined with the conditioned medium. After diluting to a KSCN concentration of 0.2 M with phosphate-buffered saline containing 0.01% sodium azide and 0.01% Tween 80 (PBAST), the preparation was clarified by centrifugation and was applied to a 3-mL column of immobilized anti-t-PA monoclonal antibody (PAM-2 Sepharose, American Diagnostica, Inc.). The t-PA was eluted with 1.5 M KSCN in PBAST and applied to a 3-mL column of Con A–Sepharose (Pharmacia). The column was washed with 0.25 M KSCN in PBAST and eluted in three stages with 6-mL aliquots of buffer. The least glycosylated t-PA eluted with 2 M KSCN, while fully glycosylated t-PA remained bound and required 0.45 M methyl α-mannoside and 2 M KSCN for elution. The majority of the t-PA recovered from the tunicamycin-treated cultures eluted with 2 M KSCN, and the t-PA from the control cultures required both methyl α-mannoside and 2 M KSCN for elution. Thus, separation on Con A–Sepharose allowed the isolation of more representative preparations of non-glycosylated, and partially, and fully glycosylated t-PA. Following elution from Con A–Sepharose, samples were concentrated by using centrifugal ultrafiltration units (Centricon 10, Amicon) and applied to an HPLC gel filtration column (GF-250, Du Pont) equilibrated with 1.6 M KSCN and 20 mM sodium phosphate, pH 6.8, containing 0.01% Tween 80. The t-PA peak was pooled, concentrated, and assayed for t-PA activity and antigen. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS–PAGE) and subsequent zymography were performed as described by Ryan et al. (1984). Bowes melanoma t-PA (American Diagnostica) and low molecular weight urokinase (Abbokinase, Abbott Laboratories) were used as standards.

Assay of t-PA Activity. The two-chain forms of the enzymes were compared in all cases. When required, samples (100–200 ng/mL) were treated for at least 1 h at room temperature with 10 ng/mL plasmin (product no. 411, American Diagnostica, Inc.) to convert any single to two-chain t-PA. Control experiments with commercial single-chain m-t-PA (product no. 111, American Diagnostica, Inc.) showed greater than 95% conversion to the two-chain form under these conditions. Enzymatic activity of t-PA was measured by the following four methods.

(*1*) *Direct Amidolytic Assay.* This uses the chromogenic peptide substrate S-2322 (H-D-Val-Gly-Arg-*p*-nitroanilide, KabiVitrum), which is cleaved directly by t-PA (Rijken & Collen, 1981). The assay was performed in 96-well microtitration plates as described previously (Parekh et al., 1989). Results are expressed as the change in absorbance (A) at 405 nm (measured in milli absorbance units) per minute (mAU/min) per μg/mL t-PA in a 100-μL reaction mixture. t-PA levels were determined in this and all subsequent assays by immunoassay (see later).

(*2*) *Unstimulated Indirect Amidolytic Assay.* This assay measures the activation of plasminogen indirectly in a reaction mixture containing 50 μg/mL Glu-type human plasminogen (product no. 400, American Diagnostica, Inc.), 1.0 mM plasmin substrate S-2251 (H-D-Val-Leu-Lys-*p*-nitroanilide, KabiVitrum), and 0–20 ng/mL t-PA sample in phosphate-buffered saline, pH 7.4 (Sigma, product 1000-3), containing 0.01% Tween 80 and 0.01% sodium azide. The reaction was carried out in 96-well microtitration plates (Immulon 1, Dynatech) at room temperature with a final reaction volume of 100 μL. Similar assays have been described by Ranby (1982) and Verheijen et al. (1982). A constant rate of plasminogen activation in this assay (i.e., constant t-PA turnover number) results in a constant increase in plasmin activity, as indicated by a constant increase in the rate of absorbance change at 405 nm (i.e., dA/dt at 405 nm). To calculate dA/dt, the absorbance of the reaction mixture was monitored by periodic readings in a plate reader (Molecular Devices Corp.) and a quadratic fit made to consecutive data triplets. dA/dt was then evaluated at the middle data point for each such set as described by others (Normann et al., 1985; Petersen et al., 1985); the slope of a plot dA/dt versus reaction time (i.e., plasmin activity versus reaction time) is proportional to t-PA activity. Plots of dA/dt versus reaction time were continuously linear over the time studied and showed no initial lag. Results are reported as the slope of the plot of dA/dt versus reaction time (in units of μAU/min^2) after normalizing to a t-PA

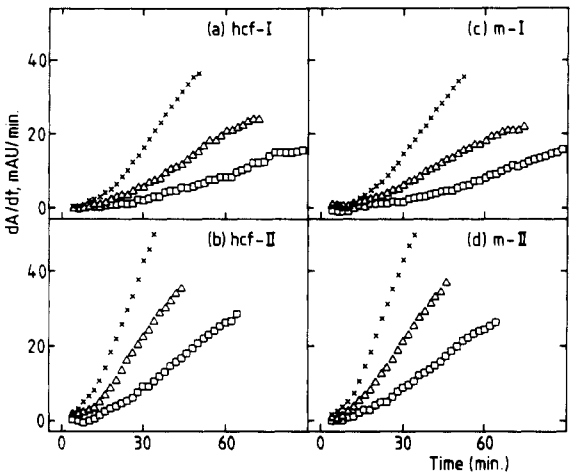

FIGURE 1: Activity of type I and type II hcf- and m-t-PA in the stimulated indirect amidolytic assay: (a) type I hcf-t-PA, (b) type II hcf-t-PA, (c) type I m-t-PA, and (d) type II m-t-PA. Assay conditions were as described under Materials and Methods, except that the final reaction volume was 200 μL. Each t-PA sample was assayed at three different final concentrations [(□) 2.5, (△) 5, and (×) 10 ng/mL]. Absorbance (405 nm) was determined every 2 min, and dA/dt was calculated as described (Materials and Methods). Points are given for the portion of the reaction course where dA/dt is directly proportional to plasmin concentration (i.e., in the range of 0.800 mAU reaction mixture absorbance, which is equivalent to 0.400 mAU for a 100-μL reaction volume).

concentration of 1 μg/mL. This slope was determined by the method of least squares.

(3) Stimulated Indirect Amidolytic Assay. Soluble fragments of human fibrinogen (t-PA stimulator, KabiVitrum) at a final concentration of 130 μg/mL were included in the above assay (2) so as to mimic the stimulatory effects of fibrin (Zamarron et al., 1984). In this assay, dA/dt was linear with reaction time only after an initial lag phase (Figure 1). The linear portion consistently occurred when the reaction mixture gave an absorbance reading in the range 100–400 mAU. In the range 0–100 mAU, a 10–20-fold lower activity was observed, and this was difficult to quantitate due to the smaller absorbance changes between subsequent readings. For this reason, results from the second, more active phase were used to calculate the slope by the method of least squares. The stimulation observed during this phase was typically 800–900 times the activity seen in the absence of fibrinogen fragments and was quite consistent for replicates within a given assay. However, there was significant variation in the stimulation observed at different times, probably due to differences in the solubility of the commercial stimulator preparation. Relative to the WHO standard, activities were consistent, regardless of the absolute level of stimulation. For this reason, results of t-PA activity in this assay are reported relative to the WHO t-PA standard (Gaffney & Curtis, 1985) in international units (IU), rather than directly in units of absorbance change.

(4) Clot Lysis Assay. In this assay, a mixture of t-PA, plasminogen, and fibrinogen was clotted by the addition of thrombin and subsequent fibrin degradation monitored by timing the passage of a glass bead through the clot (Einarsson et al., 1985). The assay was performed in 10 × 44 mm test tubes at room temperature with a final reaction volume of 1.2 mL. The final concentration of reactants was as follows: 2.5 units/mL bovine thrombin (Calbiochem), 63 μg/mL Glu-plasminogen, and 1.0 mg/mL human fibrinogen (KabiVitrum, L grade) in 0.1 M sodium phosphate buffer, pH 7.3. Four serial dilutions of t-PA were included for each sample at 2.5, 5, 10, and 20 ng/mL. A 6-mm glass bead (Kimble no. 13500) was placed on top of each clotted reaction mixture. Lysis time for each tube was determined as the time required for the glass bead to reach the bottom of the tube. Plots of log lysis time verus log t-PA concentration were linear for a given sample and parallel between samples. Activities were expressed relative to the WHO t-PA standard in international units.

t-PA Binding to Fibrin during Fibrin Clot Formation. This was determined essentially as described by Rijken et al. (1982). The following were mixed in a 1.5-mL conical centrifuge tube: t-PA (200 ng/mL), human fibrinogen as indicated (0–1 mg/mL), 1.4 mg/mL bovine serum albumin (Sigma, essentially globulin free), 0.7 mg/mL bovine γ-globulin (ICN Immunobiologicals, labile enzyme free), and 1 unit/mL bovine thrombin, in phosphate-buffered saline containing 0.01% sodium azide and 0.01% Tween 80. The final volume was 100 μL. After 1 h at room temperature, the clots were centrifuged for 1 h in a micro test-tube centrifuge at 4 °C and a sample of the supernatant was taken for measurement of t-PA antigen by ELISA. Percentage t-PA bound to the clot was calculated relative to control incubations not containing fibrinogen, but similarly prepared and processed. Human fibrinogen was rendered plasminogen free by passage over lysine–Sepharose (Pharmacia) (Deutsch & Mertz, 1970) prior to use in this assay. t-PA antigen was determined by using a commercially available ELISA kit (American Diagnostica, Inc.).

Determination of Kinetic Parameters. Kinetic parameters for the activation of Glu-plasminogen by t-PA in the indirect amidolytic assays were determined by varying the concentration of Glu-plasminogen. In the stimulated assay, the concentration of Glu-plasminogen was varied from 0.0108 to 1.08 μM (1–100 μg/mL) with t-PA held constant at about 10 ng/mL. V_{max} and K_m were determined according to the iterative method of Cleland (1967). In all cases the standard errors of the values determined in this way were less than 10% of the magnitude of the measured constants. In the unstimulated assay the concentration of Glu-plasminogen was varied from 0.215 to 21.5 μM (20–2000 μg/mL) with t-PA held constant at about 20 ng/mL. In this range of plasminogen concentrations, no departure from first-order kinetics was observed and the first-order rate constant, V_{max}/K_m, was determined from the slope of a plot of activity versus plasminogen concentration, as calculated by the method of least squares ($r > 0.998$ in all cases). V_{max} values (in units of μAU/min^2) are expressed in terms of moles of plasmin formed per second per mole of t-PA (the catalytic rate constant, k_{cat}, having units of inverse seconds) and were determined after control experiments in which the absorbance increase from known amounts of plasmin (American Diagnostica, product no. 411) was determined. Molecular weights of 93K, 85K, and 65K for Glu-plasminogen, plasmin, and t-PA, respectively, were used to calculate molar protein concentrations.

RESULTS

Activity of Type I and Type II Forms of hcf- and m-t-PA. The activities of these forms of t-PA in the four different assays are summarized in Table I. When these data are examined, it should be remembered that the stimulated and unstimulated indirect amidolytic activities are not expressed in the same units (see Materials and Methods). Thus, Table I alone cannot be used to determine the extent to which a given t-PA activity is increased by fibrinogen fragments. However, it does provide a comparison of the activity of each t-PA species by any one of the four methods employed. In this regard, there is little or no difference between the t-PA species when their activities are determined by either the direct or unstimulated amidolytic assays. However, in the presence of soluble fibrinogen fragments type II hcf-t-PA was 2.2 times more active than type

Table I: Specific Activity of Type I and Type II Forms of hcf- and m-t-PA in Four Different Enzymatic Assays

	assay[a]											
	direct amidolytic [mAU/[min·(μg/mL)]]			unstimulated indirect amidolytic [mAU/[min²·(μg/mL)]]			stimulated indirect amidolytic (IU/μg)			clot lysis (IU/μg)		
t-PA sample	mean	SD	n	mean	SD	n	mean	SD	n	mean	SD	n
hcf-t-PA (unfractionated)	7.0	0.5	5	81	9	6	374	40	5	325	26	3
hcf-t-PA type I	10.1	0.4	6	92	16	6	316	20	4	363	13	3
hcf-t-PA type II	9.1	0.6	5	93	10	6	693	49	4	459	29	3
m-t-PA (unfractionated)	10.2	0.6	5	93	12	6	560	43	4	495	42	3
m-t-PA type I	10.5	1.1	5	86	16	6	310	70	5	479	49	3
m-t-PA	10.3	0.7	6	110	18	6	754	78	4	587	42	3

[a] Assay methods and definition of units in each assay are as described under Materials and Methods.

I hcf-t-PA, and type II m-t-PA was 2.4 times more active than type I m-t-PA. Type I hcf-t-PA showed a similar activity to type I m-t-PA, while type II hcf-t-PA was less active than type II m-t-PA. These activities are determined during the steady state of the stimulated indirect amidolytic assay which is preceded by a significant lag phase. Type I t-PA shows a significantly longer lag phase (Figure 1) than the corresponding type II t-PA, indicating a lower plasminogenolytic activity of type I t-PA during the initial as well as during the steady-state period.

The reason for the biphasic nature of this assay was investigated. In preliminary tests it was found that pretreatment of the fibrinogen fragment stimulator with plasmin eliminated the lag period and gave an activity identical with that observed during the second phase without plasmin pretreatment (data not shown). It was concluded that plasmin, generated during the first phase of the assay, cleaved the stimulator and generated fragments with a more potent stimulatory effect. Others have observed the biphasic nature of such reactions stimulated by fibrinogen fragments (Jeremiah et al., 1985; Beckmann et al., 1988) or fibrin (Norrman et al., 1985) and likewise concluded its cause was digestion of the stimulator by plasmin. The stimulated indirect amidolytic assay, therefore, measures activity in the presence of plasmin-digested fibrinogen fragments. The specific fragments and cleavages involved, and the reason for this increase in stimulatory activity, have not been investigated, but the interaction of newly formed C-terminal lysine and arginine residues with the Kringle-associated lysine-binding sites on t-PA (van Zonneveld et al., 1986) and plasminogen (Castellino, 1981) may certainly be an important factor.

In the fibrin-stimulated clot lysis assay, however, the type II forms were only 23–26% more active than the corresponding type I t-PA forms. Interestingly, the m-t-PA type I and type II species were significantly more active than the corresponding hcf-t-PA forms in the clot lysis assay (by 30%), while there was little difference between them in the stimulated indirect amidolytic assay. These latter results are not considered to be conflicting, since the stimulated indirect amidolytic assay and the clot lysis assays are not identical. The stimulated indirect amidolytic assay, as described here, measures t-PA activity not in the presence of native stimulator, but rather *after* degradation by plasmin of the fibrinogen fragment stimulator. The fibrin clot lysis assay is influenced by a variety of different factors including the activity of t-PA during the initial pre-steady-state period, binding of t-PA and plasminogen to a fibrin clot (rather than to soluble fibrinogen fragments), and competition between the fibrin clot and fibrin products for the available t-PA. All three of these events are not only detected during the stimulated indirect amidolytic assay but may be of differing relative importance for the different forms of t-PA.

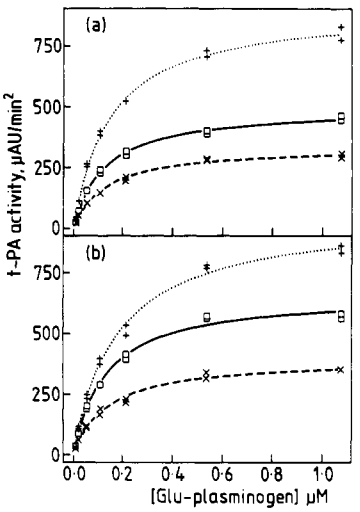

FIGURE 2: Relationship of t-PA activity to Glu-plasminogen concentration in the presence of fibrinogen fragment stimulator. Results are shown for unfractionated (□), type I (×), and type II (+) t-PA from human colon fibroblasts (a) and Bowes melanoma cells (b). Final t-PA concentrations in the reaction mixtures (ng/mL) were as follows: unfractionated hcf-t-PA (10.0), type I hcf-t-PA (9.3), type II hcf-t-PA (10.5), unfractionated m-t-PA (10.2), type I m-t-PA (10.7), and type II m-t-PA (10.7). Lines indicate the best fit of these data to the Michaelis–Menten equation, by using the iterative method of Cleland (1967).

The activity of the unfractionated hcf-t-PA is lower in all four assays than the average of the activities of the type I and type II forms. When the fractions for type I and type II hcf-t-PA were pooled following lysine–Sepharose chromatography, lower activity variants present in the bulk preparation were excluded. For example, the prominent shoulder of t-PA activity which precedes the hcf-t-PA type I fractions (Parekh et al., 1989) was significantly less active than type I in both the stimulated indirect amidolytic and clot lysis assays (data not shown). The carbohydrate or other structural features which might correlate with this lower activity remain to be investigated.

Kinetic Parameters of the Activation of Glu-Plasminogen by Type I and Type II Forms of hcf- and m-t-PA. Figures 2 and 3 show the relationship of activity to plasminogen concentration obtained for the hcf- and m- t-PA samples in the indirect amidolytic assay in the presence and absence of fibrinogen fragments, respectively. In the presence of stimulator (Figure 2) Michaelis–Menten kinetics were followed, allowing K_m, K_{cat}, and K_{cat}/K_m to be calculated. These data are summarized in Table II and indicate that, in the presence

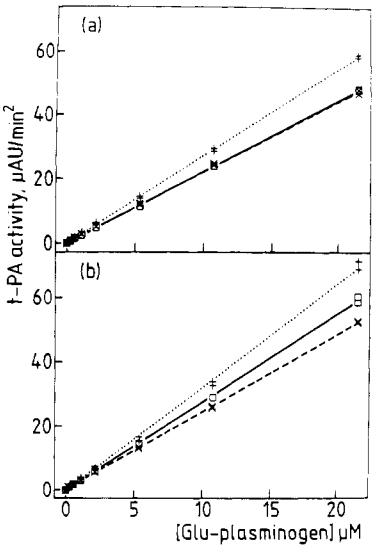

FIGURE 3: Relationship of t-PA activity to Glu-plasminogen concentration in the absence of any stimulator. Results are shown for hcf-t-PA (a) and m-t-PA (b) as indicated in Figure 2. The final t-PA concentrations in each of the reaction mixtures were twice those given in Figure 2. Lines indicate the best linear fit to these data by the method of least squares.

Table II: Kinetic Parameters for Type I and Type II Forms of hcf- and m-t-PA[a]

t-PA and conditions	K_m (μM)	K_{cat} (s^{-1})	K_{cat}/K_m (μM^{-1} s^{-1})
with stimulator			
hcf-t-PA (unfractionated)	0.13	0.107	0.82
hcf-t-PA type I	0.13	0.078	0.60
hcf-t-PA type II	0.15	0.186	1.23
m-t-PA (unfractionated)	0.13	0.140	1.08
m-t-PA type I	0.14	0.080	0.57
m-t-PA type II	0.18	0.200	1.11
without stimulator			
hcf-t-PA (unfractionated)	>22[b]	–[b]	0.00013
hcf-t-PA type I	>22		0.00014
hcf-t-PA type II	>22		0.00015
m-t-PA (unfractionated)	>22		0.00016
m-t-PA type I	>22		0.00013
m-t-PA type II	>22		0.00018

[a] Assays were performed as described under Materials and Methods. [b] There was no departure from apparent first-order kinetics up to a concentration of Glu-plasminogen of 21.5 μM.

of soluble fibrinogen fragments, the K_m for plasminogen is essentially the same for all forms of t-PA, but the K_{cat} is 2.4- or 2.5-fold greater for type II t-PA than for the corresponding type I form. In the absence of stimulator, there was no evidence of departure from first-order kinetics over the range of Glu-plasminogen concentrations investigated (Figure 3). Therefore, K_{cat}/K_m was calculated from the slope of the plot of t-PA activity versus Glu-plasminogen concentration (Figure 3), as has been done by others in this situation (Rijken et al., 1982; Little et al., 1984; Petersen et al., 1985). The results are summarized in Table II and indicate little difference in the activities of the various t-PA forms in the absence of any stimulator. From the data summarized in Table II, it can be calculated that, at a physiological concentration of Glu-plasminogen (2 μM), the type I t-PA forms are stimulated 260- (hcf-t-PA) and 290-fold (m-t-PA), and the type II t-PA forms 580- (hcf-t-PA) and 510-fold (m-t-PA), by the fibrinogen fragment stimulator. The K_m and K_{cat} values determined in this study are similar in order of magnitude to those determined by others for unfractionated t-PA in this type of assay (Zamarron et al., 1984; Beckmann et al., 1988; Holvoet et al., 1986a; de Vries et al., 1988).

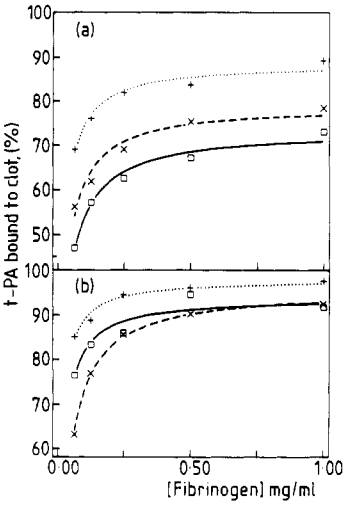

FIGURE 4: Binding of type I and type II forms of hcf- and m-t-PA to fibrin. Results are shown for unfractionated (□), type I (×), and type II (+) t-PA from human colon fibroblasts (a) and Bowes melanoma cells (b). Each point represents the mean of four separate determinations. With one exception (m-t-PA at 1.0 mg/mL fibrin), all mean values for type I and type II forms of a given t-PA species and fibrin concentration were significantly different at 95% level of confidence using Student's two-tailed *t* test. Lines indicate the best fit of these data to a rectangular hyperbola using the iterative method of Cleland (1967).

Binding of Type I and Type II Forms of hcf- and m-t-PA to Fibrin. The relative affinity for fibrin of each t-PA sample was determined by measuring the amount of t-PA remaining in the soluble phase when a mixture of fibrinogen and t-PA was clotted with thrombin. Figure 4 shows the relationship obtained between initial fibrinogen concentration and the relative amount of t-PA bound to the resulting fibrin clot, as calculated by difference. For both hcf- and m-t-PA, the type II form binds to a significantly greater extent to fibrin, at the concentrations tested, than does the type I form. It also appears that the m-t-PA forms bind more tightly than the comparable hcf-t-PA forms. The relative affinity for fibrin of the six t-PA forms (Figure 4) correlates well with their activity in the clot lysis assay, as summarized in Table I (r = 0.98, using the fibrin binding data obtained at 1 mg/mL), but does not correlate significantly with their activity in the stimulated indirect amidolytic assay (r = 0.62).

Unfractionated hcf-t-PA bound significantly less to fibrin than did either the type I or type II forms isolated from it. As mentioned above, this seemingly anomalous behavior, together with the lower than expected in vitro assay results (Table I), may be explained by the presence of fractions with lower fibrin affinity and lower specific activity in the unfractionated t-PA, which were not included in the pools for type I and type II t-PA after lysine–Sepharose chromatography.

Activity of t-PA Isolated from Cells Treated with Tunicamycin. Human colon fibroblast and Bowes melanoma cells were incubated with B_2-tunicamycin, and the t-PA was isolated from cells and conditioned medium (see Materials and Methods). Such t-PA was compared with t-PA isolated in a similar manner from control clusters incubated without B_2-tunicamycin. As judged by the relative incorporation into the isolated t-PA of label from [^{3}H]mannose present in the culture medium, t-PA derived from tunicamycin-treated cultures contained about one-tenth of the mannose of t-PA from control cultures (Table III). Incorporation of [^{3}H]leucine into t-PA was however not decreased as a result of tunicamycin treatment. Further, most of the active t-PA obtained from tuni-

Table III: Activity of t-PA Isolated from Control and Tunicamycin-Treated Cells

t-PA source	assay[a] direct amidolytic [mAU/[min·(μg/mL)]]	unstimulated indirect amidolytic [mAU/[min²·(μg/mL)]]	stimulated indirect amidolytic (IU/μg)	[³H]mannose incorpn (cpm/μg)	[³H]leucine incorpn (cpm/μg)
human colon fibroblast					
control[b]					
prepn 1	6.6	96 ± 7	354 ± 11	14680	
prepn 2	7.4	94 ± 7	472 ± 23		3210
tunicamycin treated					
prepn 1	6.3	148 ± 9	531 ± 58	1930	
prepn 2	7.8	257 ± 22	837 ± 71		4660
Bowes melanoma					
control[b]					
prepn 1		79 ± 13	309 ± 17	19120	
prepn 2		90 ± 11	349 ± 28		3110
tunicamycin treated					
prepn 1		250 ± 19	700 ± 46	2320	
prepn 2		393 ± 5	771 ± 22		4020

[a] See Materials and Methods for assay methods and for definition of assay units. Where indicated, results are presented as mean values ± the standard error of the mean. [b] This refers to t-PA isolated from cell cultures incubated in the absence of tunicamycin.

camycin-treated cultures migrated with a relative molecular weight of 59K, as judged by SDS–PAGE zymography (data not shown). This is the expected molecular weight of aglycosyl t-PA (Pennica et al., 1983).

The activity of the t-PA preparations derived from tunicamycin-treated cultures was significantly greater than that of the t-PA from control preparations, when measured in both the stimulated and unstimulated indirect amidolytic assays (Table III). The increase in activity was from 2- to 4-fold in the absence of stimulator, and about 2-fold in the presence of stimulator, depending on the individual preparation. Direct amidolytic assays, however, were relatively constant and showed no difference between control t-PA and that from tunicamycin-treated cultures. The constant amidolytic activities of these samples support the use of an immunoassay to normalize assay results and suggest that true activity differences (rather than different immunoassay responses) are being detected in the indirect assay.

Discussion

In a previous report (Parekh et al., 1989) the N-glycosylation at each site of type I and type II m- and hcf-t-PA was described. It was shown that there were no glycoforms in common between hcf-t-PA and m-t-PA. It is not yet possible to separate experimentally the individual glycoforms of either hcf- or m-t-PA. However, the previously defined type I and type II *sets* of glycoforms can be separated, and aglycosyl t-PA can be isolated from tunicamycin-treated cultures. A comparison of type I hcf-t-PA with type I m-t-PA, or of type II hcf-t-PA with type II m-t-PA, examines the effect of differences in oligosaccharide structure on t-PA activity, while a comparison of type I, type II, and aglycosyl t-PA's examines the influence of the number of N-linked oligosaccharides associated with t-PA on its activity. The results of these comparisons suggest that both the occupancy and nature of oligosaccharides at the N-glycosylation sites influence the in vitro activity of t-PA.

Comparison of type I and type II t-PA, and aglycosyl t-PA isolated from cells cultured in the presence of tunicamycin, shows that the number of sequons occupied affects t-PA activity. By use of data drawn from Tables I–III, the influence of sequon occupancy on activity in the indirect amidolytic assay is summarized in Figure 5. It is seen that carbohydrate does affect the stimulation of t-PA by fibrinogen fragments and

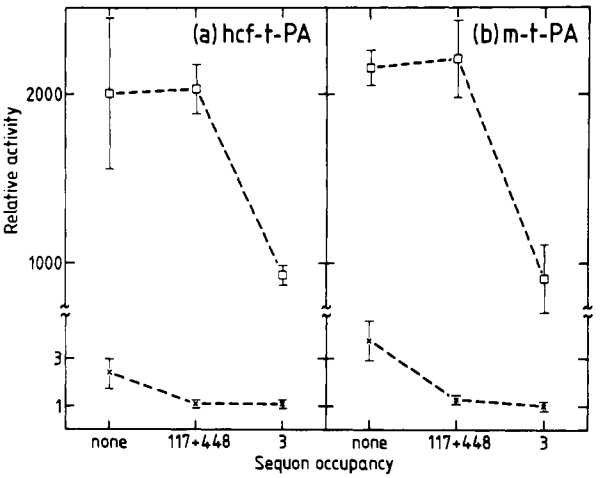

FIGURE 5: Influence of the occupancy of N-glycosylation sequons on the relative activity of (a) hcf-t-PA and (b) m-t-PA measured by the indirect amidolytic assay in the presence (□) and in the absence (×) of fibrinogen fragments. Assay values taken from Tables I and III are expressed relative to the unstimulated activity of type I m-t-PA (of relative activity = 1). The activity of t-PA from tunicamycin-treated cultures (Table III) is used to represent unglycosylated t-PA (no occupied sequons), and the activities of type I and type II t-PA (Table I) represent, respectively, fully glycosylated t-PA (all three sequons occupied) and t-PA glycosylated at sites Asn-117 and -448, but not -184 (117 + 448). Since the stimulated and unstimulated indirect amidolytic activities reported in Tables I and III are expressed in different units, a relative stimulated activity of 908 for type I m-t-PA was calculated by using the kinetic constants given in Table II and a Glu-plasminogen concentration of 0.54 μM (50 μg/mL), i.e., the concentration employed for the indirect amidolytic assay in Tables I and III. The other stimulated assay values were then expressed relative to this value. Although the type I m-t-PA constants were used for this procedure, essentially identical results are obtained if the kinetic constants for any of the other t-PA species are employed. Error bars indicate the standard deviation or, in the case of unglycosylated t-PA (none), the range of the two preparations analyzed in Table III. For clarity, a dashed line connects activities obtained under the same assay conditions.

that the three glycosylation sites are not equivalent in this. Glycosylation at Asn-117 and Asn-448 reduces unstimulated activity 2-fold or more, but further glycosylation at Asn-184 has no effect. Glycosylation at Asn-117 and Asn-448 does not change the stimulated activity, but further glycosylation at Asn-184 reduces this activity by a factor of about 2 and also decreases, to a small but significant extent, fibrin binding

and fibrin clot lysis activity (Table I and Figure 3). The nature of the N-linked oligosaccharides at a given site also influences activity. Type I m-t-PA was 32% more active than type I hcf-t-PA in the clot lysis assay, and 18% more of type I m-t-PA was bound to a forming fibrin clot than type I hcf-t-PA. Similarly, type II m-t-PA was 28% more active, and 10% more was bound, when compared to type II hcf-t-PA. Since this difference exists for both type I and type II species, and since Asn-117 is associated with very similar oligosaccharides in both hcf-t-PA and m-t-PA, it is likely that the more biosynthetically complete galactosylated and sialylated structures at site Asn-448 in hcf-t-PA (Parekh et al., 1989) are responsible for this reduction in activity and fibrin binding.

No effect of glycosylation was noted on the direct amidolytic activity. Apparently, the oligosaccharide structures are located so as not to interfere with binding and hydrolysis of a low molecular weight substrate. It has been shown that the amidolytic activity of the isolated t-PA B chain is identical with that of intact two-chain t-PA (Holvoet et al., 1986b; Rijken & Groeneveld, 1986), implying that the direct amidolytic activity is primarily indicative of active site concentration. One-chain t-PA has a lower direct amidolytic activity than two-chain t-PA (Ranby et al., 1982), but the samples in the present study were all in the two-chain form.

The glycosylation sites of t-PA are located in the first Kringle (Asn-117), the second kringle (Asn-184), and the serine protease domain (Asn-448). With plasminogen as substrate, the unstimulated indirect amidolytic activity decreased when Asn-117 and Asn-448 were glycosylated, compared to aglycosyl t-PA (Figure 4). Oligosaccharide at Asn-448 may therefore hinder binding of the large, 93K plasminogen molecule. Further glycosylation at a site removed from the serine protease domain (Asn-184) leaves the unstimulated activity unchanged, but does depress stimulated activity (Figure 4). To understand this effect of glycosylation at Asn-184, the domains involved in the stimulation of t-PA activity should be considered. When t-PA is stimulated by fibrin or fibrinogen fragments, biphasic kinetics are observed (Norrman et al., 1985; Beckmann et al., 1988). The slower, first phase is thought to be stimulated by the binding of native fibrin to the fibronectin finger domain of t-PA, while in the faster, second phase, plasmin-degraded fibrin interacts with the lysine-binding site on the second Kringle domain (van Zonneveld et al., 1986). Plasmin-degraded fibrinogen fragments are the stimulator in the stimulated indirect amidolytic assay (see Materials and Methods), while stimulation by native fibrin is certainly important for clot lysis activity. The large, negative effect of glycosylation at Asn-184 during both the initial and steady-state periods of the stimulated indirect amidolytic activity, and the smaller effect on clot lysis activity, is therefore consistent with oligosaccharide at Asn-184 weakening the interaction of the second Kringle with plasmin-degraded stimulator. Binding of native fibrin by the finger domain and, hence, clot lysis activity should be less affected by glycosylation at Asn-184, and this is indeed observed. However, the influence of the nature of the oligosaccharide at Asn-448 on both fibrin binding and clot lysis activity suggests that the finger domain and the site Asn-448 oligosaccharide may be in close proximity in the three-dimensional t-PA structure.

The effects of N-glycosylation on the in vitro enzymatic activities of t-PA provide some insight into the physiological consequences of N-glycosylation of the t-PA polypeptide. The reduction in the unstimulated t-PA activity caused by N-glycosylation at Asn-117 and Asn-448 would reduce basal, clot-independent, plasminogen activation to a level lower than that due to unglycosylated t-PA. Since t-PA-initiated fibrinolysis in vivo is a multistep cascade subject to control by several circulating components, the differences in activity detected in vitro would be much greater in vivo (Stadtman et al., 1981). Further, the type I and type II t-PA studied here are each a mixture of many different glycoforms, and the activity of each type I and type II t-PA is a composite of the activity of each constituent glycoform. It is therefore expected that much greater effects of N-glycosylation would be seen with pure glycoforms. For example, the shoulder of hcf-t-PA activity that elutes before type I from lysine–Sepharose [Parekh et al. (1989) and unpublished observations] has a specific activity about one-fifth of that of type II in the stimulated amidolytic assay. Further, two-chain m-t-PA was fractionated on lysine–Sepharose, and the minor fractions were pooled and analyzed separately from the major type I and type II m-t-PA [Parekh et al. (1989) and unpublished observation]. Marked differences between individual pools were seen in both the initial and steady-state activities when measured by the stimulated indirect amidolytic assay. These pools are differently N-glycosylated (unpublished observation), indicating that N-glycosylation may serve to create "fast"- and "slow"-acting forms of the t-PA polypeptide. In a mixture of glycoforms of t-PA, therefore, individual glycoforms may not all be simultaneously active, but may rather act in sequence, resulting in a gradual initiation of fibrinolysis. This may help in protecting newly formed fibrin while still providing for the production of sufficient plasmin for eventual clot lysis. In short, the diversification of the t-PA polypeptide into a set of glycoforms, some of which have a shorter lag phase and higher steady-state activity in the presence of stimulator ("fast acting") and others a longer lag phase and lower steady-state activity ("slow acting"), and differences in binding of fibrin, fibrinogen fragments, and plasminogen may help to balance the complex and conflicting physiological requirements for both initial clot persistence and final clot lysis.

Several previous reports have indicated that oligosaccharides might affect t-PA activity. Opdenakker et al. (1986) found significant changes in specific activity after t-PA was treated with exoglycosidases. Using a clot lysis assay, Einarsson et al. (1985) found that type II m-t-PA was 50% more active than type I m-t-PA, while Ranby et al. (1982) reported an 8–14% difference. These results are in general agreement with those reported here. However, Zamarron et al. (1984) report equal K_{cat} values for type I and type II recombinant t-PA in the presence of fibrinogen fragments (0.24 and 0.25 s^{-1}, respectively), but a 4-fold difference in the K_{cat}/K_m (0.008 and 0.0035 μM^{-1}, respectively) in the absence of stimulator. These results are contrary to those reported in Table II, where unstimulated activity is similar, but stimulated activity increases for type II. Although differences in sample source or assay conditions (Wittwer et al., 1987) may account for this disagreement, it is important to note that Zamarron et al. did not employ an activity-independent method to determine enzyme concentration. In their comparison of type I and type II t-PA concentration was adjusted to equal activity in a separate fibrin plate assay. Thus, the actual t-PA concentrations were not necessarily equivalent and only K_m-derived, but not K_{cat}-derived, values would be accurate. In the present study, t-PA activity levels were normalized on the basis of antigen levels determined by ELISA. This procedure was justified by the equal amidolytic activities of the type I and type II t-PA (Table I) and t-PA from tunicamycin-treated and control cells (Table III).

Comparing t-PA derived from Bowes melanoma cells cultured in the presence or absence of tunicamycin, Rijken et al. (1985) concluded that N-glycosylation has no effect on fibrin binding or fibrin-stimulated plasminogen activation. The t-PA in their study, however, was not purified, and the extent to which N-glycosylation was inhibited was not demonstrated. This is important because nonglycosylated t-PA may be inefficiently secreted (Little et al., 1984). Little et al. (1984) compared native m-t-PA and m-t-PA treated with endoglycosidase H and found no difference in fibrin-stimulated activity. However, this glycosidase releases only exposed, high-mannose oligosaccharides (Hubbard & Ivatt, 1981). Owing to the presence of primarily complex oligosaccharides at sites Asn-184 and Asn-448 in m-t-PA (Parekh et al., 1989), endoglycosidase H treatment could at best result in only a partially deglycosylated molecule. The present work is the first to actually purify nonglycosylated t-PA and to measure its activity both in the presence and in the absence of stimulator.

Finally, it is worth noting that type I and type II plasminogen, which also differ with respect to the presence of an N-linked oligosaccharide (at Asn-228) in type I but not in type II, also differ in their affinity for fibrin and α_2-antiplasmin (Lijen et al., 1981). Type II plasminogen binds more tightly to both than does type I plasminogen. Examples of the effects of the degree of sequon occupancy and the nature of the oligosaccharide at a given sequon on glycoprotein function have recently been reviewed (Rademacher et al., 1988) and include cell adhesion mediated by the embryonic and adult forms of mouse NCAM (Rutishauser et al., 1988), factor IX (Chavin & Weidner, 1984), antithrombin IIIα and β (Peterson & Blackburn, 1985), and factor V (Bruin et al., 1987). That subtle changes in N-glycosylation may, in a physiological context, be of great importance is suggested by a comparison of native fibrinogen and that from patients with liver disease associated dysfibrinogenemia (Martinez et al., 1983).

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