N-Glycosylation and in vitro enzymatic activity of human recombinant tissue plasminogen activator expressed in Chinese hamster ovary cells

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N-Glycosylation and in Vitro Enzymatic Activity of Human Recombinant Tissue Plasminogen Activator Expressed in Chinese Hamster Ovary Cells and a Murine Cell Line

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ABSTRACT: To probe the effects of N-glycosylation on the fibrin-dependent plasminogenolytic activity of tissue-type plasminogen activator (t-PA), we have expressed a human recombinant t-PA (rt-PA) gene in Chinese hamster ovary (CHO) cells and in a murine C127 cell line. The resulting rt-PA glycoproteins were isolated and their associated N-linked oligosaccharide structures determined by using a combination of high-resolution Bio-Gel P-4 gel filtration chromatography, sequential exoglycosidase digestion, and methylation analysis. The results show that CHO rt-PA is N-glycosylated differently from murine C127 derived rt-PA. Further, both rt-PA's are N-glycosylated differently from t-PA derived from a human colon fibroblast and the Bowes melanoma cell line (Parekh et al., 1989), confirming that N-glycosylation of the human t-PA polypeptide is cell-type-specific. Both CHO and murine rt-PA were fractionated on lysine–Sepharose chromatography. The N-glycosylation of the major forms was analyzed and their fibrin-dependent plasminogenolytic activity determined by using an indirect amidolytic assay with Glu-plasminogen and a chromogenic plasmin substrate. The results suggest that the various forms of rt-PA differ from one another with respect to the kinetics of their fibrin-dependent activation of plasminogen. Together, these data support the notion (Wittwer et al., 1989) that N-glycosylation influences the fibrin-dependent catalytic activity of t-PA and that t-PA when expressed in different cell lines may consist of kinetically and structurally distinct glycoforms.

Tissue plasminogen activator (t-PA),[1] the physiologically significant initiator of thrombolysis, is a glycoprotein containing four potential N-glycosylation sites, of which either three (type I) or two (type II) are occupied (Pennica et al., 1983; Pohl et al., 1984). The normal serum level of t-PA is low (~4 μg/L of plasma) (Hamsten et al., 1985; Loscalzo & Braunwald, 1988), and its isolation from serum is complicated by the presence of an endogenous plasminogen activator inhibitor (PAI-1) (Sanzo et al., 1987). Transformed human cell lines, in particular, the Bowes melanoma line, provide enough t-PA for experimental analysis, but not for clinical applications (Rijken & Collen, 1981). For these reasons, recombinant human t-PA has been expressed in a variety of nonhuman and bacterial cell lines (Harris, 1987). Such systems are generally assumed to be faithful with respect to transcription and translation of the recombinant gene [see, however, Opdenakker et al. (1988)]. However, with respect to posttranslational modifications, recombinant DNA derived t-PA could vary greatly from the naturally occurring form. Bacterial sources that lack an N-glycosylation apparatus have not been used to isolate t-PA for therapeutic use. Expression of t-PA in *Escherichia coli* has not yielded sufficient material appropriate for clinical evaluation, as judged by in vitro activity assays.

The currently preferred cell type for expression of human t-PA and other human glycoproteins of potential therapeutic value (such as erythropoietin) is the Chinese hamster ovary (CHO) cell (Zamarron et al., 1984). Coupled with the methotrexate-amplifiable dihydrofolate reductase marker, this cell type is relatively easy to manipulate and has been successfully used to produce a variety of human glycoproteins (Gasser et al., 1982; Kaufman & Sharp, 1982; Haynes & Weissman, 1983; Scahill et al., 1983). A second, murine cell line C127, in conjunction with bovine papilloma virus based vectors, has also been used to produce such proteins (Sarvar et al., 1981; DiMaio et al., 1982; Pavlakis & Hamer, 1983). However, N-glycosylation is known to be species-specific, and also tissue- or cell-type-specific (Rademacher et al., 1988a). That is, the final N-glycosylation of a polypeptide is influenced by the cell type in which it is expressed (i.e., cellular glycotype). Therefore, expression of a polypeptide in a cell type other than that in which it is normally expressed might be expected to lead to an N-glycosylation pattern different from that of the native form. Altered N-glycosylation could disturb functions normally influenced by oligosaccharides, as well as conferring new ones, or even render the recombinant glycoprotein immunogenic by creating novel epitopes or raising the levels of ones that were previously subimmunogenic.

Tissue plasminogen activator is proving to be of therapeutic value in the treatment of thromboembolic disorders (Verstraete et al., 1985a,b; Loscalzo & Braunwald, 1988). It was initially considered to be preferable to streptokinase and urokinase because it has a higher fibrinolytic and lower fibrinogenolytic activity (Verstraete et al., 1985a,b). In clinical trials, it is at

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[1] Abbreviations: t-PA, tissue-type plasminogen activator; rt-PA, recombinant tissue-type plasminogen activator; CHO, Chinese hamster ovary; hcf, human colon fibroblast; m, Bowes melanoma; BPV, bovine papilloma virus; mMTI, mouse metallothionein I; dhfr, dihydrofolate reductase; RSV LTR, Rous sarcoma virus long terminal repeat; DMEM, Dulbecco's modified Eagle's medium; KIU, kallikrein inhibitor unit; MTX, methotrexate.

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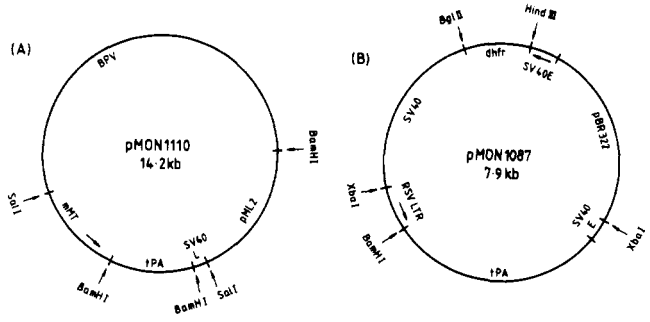

FIGURE 1: Vectors used for the expression of rt-PA in C127 and CHO cells. (A) pMON1110 for expression in C127 cells. This vector consists of the entire BPV genome cloned in the bacterial plasmid pML2. pMON1110 utilizes the mMTI promotor and SV40 late poly(A) addition site for the expression of Φ, an rt-PA cDNA fragment. (B) pMON1087 for expression in CHO cells. This vector is based on the parental plasmid pSV2 dhfr and employs the RSV LTR and the SV40E poly(A) addition site for the expression of an rt-PA cDNA.

least as effective as other thrombolytic agents or aspirin in reducing mortality after mycocardial infarction (Wilcox et al., 1988; ISIS-II Study, 1988). However, its relative side effects and overall efficacy have not been clearly distinguishable from other physiologically irrelevant thrombolytic agents such as streptokinase (Wilcox et al., 1988; Loscalzo & Braunwald, 1988). Despite the production of t-PA in several different animal cell lines, and the use of rt-PA isolated from CHO cells in the clinic (Collen et al., 1984; Browne et al., 1985a,b; Kaufman et al., 1955; Goeddel et al., 1986; Reddy et al., 1986; Sambrook et al., 1986; Weidle et al., 1987), neither its N-glycosylation nor that of any other recombinant t-PA has been defined. Such information could complement efforts to elucidate the functional behavior of a given t-PA species.

We have therefore expressed a human rt-PA gene in CHO cells and in a murine C127 cell line. The resulting rt-PA glycoproteins were isolated, their N-glycosylation was analyzed, and the in vitro activity of various forms of the rt-PA (separated by lysine–Sepharose chromatography) was assessed by using the stimulated indirect amidolytic assay. Our results show that both CHO and murine rt-PA are N-glycosylated differently with respect to each other and to t-PA secreted by normal and transformed human cell lines (Parekh et al., 1989). They support our previous conclusion that both the occupancy and nature of the oligosaccharides at N-glycosylation sites of t-PA can influence its fibrin-dependent activation of plasminogen (Wittwer et al., 1989).

Materials and Methods

Expression of rt-PA in CHO Cells and Mouse C127 Cells. The vectors for the expression of rt-PA are shown in Figure 1. pMON1110 is a bovine papilloma virus (BPV) based vector for expression in mouse C127 cells (Figure 1A). This vector consists of the entire BPV genome cloned into the bacterial plasmid pML2. pMON1110 also contains the mouse metallothionein I (mMTI) promotor and the SV40 late poly(A) addition site to direct the expression of a 1.7-kb HeLa cell t-PA cDNA fragment. A full-length cDNA from which this fragment was prepared was kindly provided by Dr. Ned Waller, Rockefeller University. Figure 1B shows the structure of the dihydrofolate reductase (dhfr) vector for expression of t-PA in CHO cells. pMON1087 is based on the parental vector pSV2dhfr (Subramani et al., 1981). The t-PA expression casette in pMON1087 consists of the Rous sarcoma virus long terminal repeat (RSV LTR), a 2.2-kb HeLa cell t-PA cDNA fragment, and the SV40 early poly(A) addition site.

Mouse C127 cells (ATCC No. CRL 1616) and CHO DBX-11 cells (Urlaub & Chasin, 1980) were both transfected by the method of Wigler et al. (1979). In the case of the C127 cells pMON1110 was cotransfected with pSV2neo (Southern & Berg, 1982), and positive transfectants were selected by resistance to the antibiotic G418. Transfectants of the dhfr-negative CHO DBX-11 cells were selected for a conversion to the dhfr-positive phenotype by pMON1087. The transfected C127 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and 50 kallikrein inhibitor units (KIU)/mL aprotinin. The CHO cells were grown in DMEM containing 10% dialyzed fetal bovine serum, 50 KIU/mL aprotinin, and 150 μg/mL proline. G418-resistant C127 cell colonies were picked and expanded. The rt-PA expression level of each was determined by ELISA (American Diagnostica); the line with the highest expression level, 1110-3, was subcloned by limiting dilution. Subcloning yielded a line 1110-3S4, which was used for scale-up. The positive CHO cell transfectants were picked, and the resulting lines were assayed for basal rt-PA expression by ELISA. Candidate lines were then subjected to amplification with the folate antagonist methotrexate (MTX) (Schimke et al., 1984). After each level of MTX amplification, rt-PA expression levels were determined. After exposure to 100 μM MTX, line 1087-20 was chosen for scale-up.

Purification of CHO and C127 Cell Derived rt-PA. The C127 cell line 1110-3S4 and the CHO cell line 1087-20 were both scaled up in a combination of Nunc cell factories, roller bottles, and spinner cultures. Both cell lines were grown in DMEM containing 10% fetal bovine serum and 50 KIU/mL aprotinin. CHO cell growth media was also supplemented with 150 μg/mL proline and 10 μM MTX. After the cells had reached confluency, the growth medium was removed and the cells were washed with phosphate-buffered saline. Each culture was then replenished with DMEM containing 0.25% lactalbumin hydrolysate. Conditioned medium was harvested every 2 days. The medium was concentrated by ultrafiltration and adjusted to 0.5 M NH_4HCO_3 and 1% Triton X-100. Following centrifugation the supernatant was loaded onto an affinity column consisting of *Erythrina caffra* trypsin inhibitor conjugated to Sepharose 4B (Heussen et al., 1984). Following washing, the column was eluted with 2.5 M KSCN, and the purified t-PA was dialyzed against 1 M NH_4HCO_3.

Lysine–Sepharose Chromatography. Eight hundred micrograms of two-chain t-PA at 1 mg/mL in 1 M ammonium bicarbonate was diluted with 1.2 mL of equilibration buffer (10 mM sodium phosphate buffer, pH 8.0, containing 0.15 M KSCN, 0.001% Tween 80, and 25 KIU of aprotinin/mL) and applied to a lysine–Sepharose column (45 × 0.9 cm), equilibrated with the same buffer at 4 °C. Lysine–Sepharose (Sigma) had coupled ligand at a density of 4 μM/mL of gel. The t-PA was applied to the column and washed with 35 mL of equilibration buffer, at a flow rate of 5.5 mL/h, and 1.0-mL fractions were collected. The bound t-PA was eluted with a linear gradient of 0–0.25 M L-arginine in equilibration buffer (160 mL), and fractions were collected. The column eluate was monitored by using the direct amidolytic assay. Aliquots were assayed in 96-well microtiter plates using a Molecular Devices kinetic microplate reader.

Assay of rt-PA Activity. (1) Direct Amidolytic Assay. The direct amidolytic assay was performed at 37 °C by using the synthetic chromogenic substrate S-2288 (KabiVitrum) in a reaction mixture consisting of 1 mM S-2288 in 0.1 M Tris buffer, pH 7.5 (at 25 °C), containing 0.1% Tween 20 (v/v),

and the absorbance monitored at 405 nm. t-PA was converted to 100% two-chain form prior to being assayed.

(*2*) *Stimulated Indirect Amidolytic Assay*. The stimulated indirect amidolytic assay was performed at 37 °C in a reaction mixture consisting of 0.13 μM Glu-type human plasminogen (KabiVitrum), 0.7 mM plasmin substrate S-2251 (KabiVitrum), 0.6 μM soluble fragments of human fibrinogen, and rt-PA (of activity defined by using the direct amidolytic assay with S-2288) in 0.1 M Tris buffer, pH 7.5, containing 0.1% Tween 20 and monitored at 405 nm on a Pye Unicam 8620 spectrophotometer. A plot of the $dA(405\ nm)/dt$ versus time represents the plasmin activity versus reaction time. Soluble fibrin fragments were produced by cleavage of human fibrinogen with cyanogen bromide, as described by Verheijen et al. (1983). The concentration equivalents of fibrin fragments were estimated by assuming that one soluble fibrin fragment is generated from each fibrinogen molecule.

Oligosaccharide Analysis. The N-linked oligosaccharides associated with a particular rt-PA glycoprotein were released, isolated, and labeled as described previously (Parekh et al., 1989). Structural analysis of the oligosaccharides was performed by a combination of high-resolution Bio-Gel P-4 gel filtration chromatography, sequential exoglycosidase digestion, and methylation analysis. Standard oligosaccharides, glycosidases, conditions of digestion of oligosaccharides with glycosidases, and the protocol for methylation analysis were the same as those described previously (Parekh et al., 1989).

Results

Expression and Purification of rt-PA. Both the BPV vector/C127 cell and the dhfr vector/CHO cell systems were capable of generating cell lines expressing significant levels of rt-PA. The highest C127 cell line, 1110-3S4, had an expression level of approximately 20 μg/(10^6 cells·24 h). This expression level yielded conditioned media containing 16 μg/mL rt-PA when scaled up. The best CHO cell line, 1087-20, expressed rt-PA at a somewhat lower level, namely, 10 μg/(10^6 cells·24 h). During the scale-up of this cell line, the conditioned medium contained 13 μg/mL rt-PA. The production levels of both lines remained relatively stable during the extended scale-up period.

The *E. caffra* trypsin inhibitor affinity column provided an extremely efficient method for the isolation of rt-PA. The final recoveries were approximately 95%. Gel analysis of the final protein showed it to be pure to the limits of detection (data not shown). The rt-PA derived from the murine C127 cell line was all in the single-chain form in structure, while 40% of the rt-PA derived from CHO cells was in the single-chain form. Prior to fractionation on lysine–Sepharose both the murine and CHO t-PA were converted to 100% two-chain t-PA (SDS gel not shown) by incubating t-PA (100–200 ng/mL) for at least 1 h at room temperature with 10 ng/mL plasmin.

Separation of t-PA by Lysine–Sepharose Chromatography. While arginine–Sepharose chromatography has been widely used to fractionate t-PA into its type I and type II forms, we consistently find such separation to be more efficient and reproducible using lysine–Sepharose, in agreement with Einarsson et al. (1985). As expected, two major peaks (fractions B and C, respectively), corresponding to type I and type II forms [as judged by nonreducing SDS–PAGE (not shown)], were obtained from both CHO and murine rt-PA (Figures 2 and 3). In addition, a shoulder on the murine, and CHO, rt-PA was detected (termed type A); a similar result was obtained previously during fractionation of t-PA derived from a human colon fibroblast cell line (Parekh et al. 1989), and

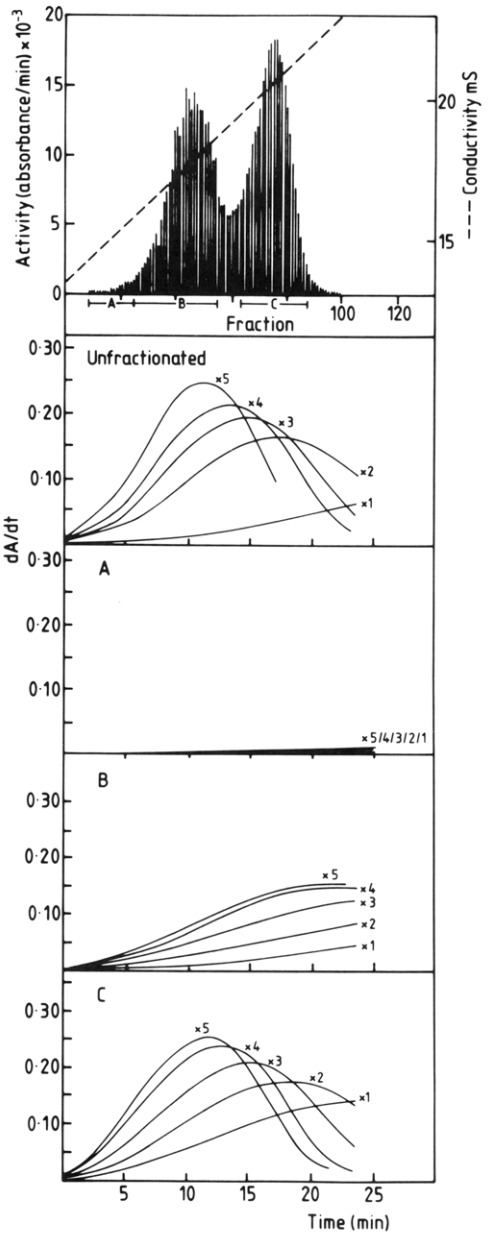

FIGURE 2: CHO rt-PA was fractionated by lysine–Sepharose chromatography (see Materials and Methods), and t-PA activity in each fraction was monitored by the direct amidolytic assay. Fractions were pooled as indicated (A–C), and following convention, peak B is designated as type I and peak C as type II CHO rt-PA. SDS–PAGE analysis under nonreducing conditions confirmed this (not shown). The activity of each pool of CHO rt-PA was measured directly with S-2288, and each fraction was then assayed in the stimulated indirect amidolytic assay by using equivalent activity of rt-PA and dA/dt monitored continually with reaction time in a Pye Unicam 8620 spectrophotometer. A similar concentration of fibrin fragments was used in A–C. The lowest concentration (1×) contains approximately 100 ng of t-PA/mL of reaction mixture. The activities of increasing levels of rt-PA are also plotted (2×–5×).

a late fraction D was also found for the CHO [similar to that found for Bowes melanoma t-PA (Parekh et al., 1989)]. Fractions were pooled as indicated (Figures 2 and 3, top panels) and assayed in the stimulated indirect amidolytic assay. Equivalent amounts of t-PA as determined by the direct amidolytic assay (using S-2288 as substrate) were compared. The correlation between direct amidolytic activity and protein content has been examined previously (Wittwer et al., 1989).

Activity of Individual CHO and Murine rt-PA Fractions in the Stimulated Indirect Amidolytic Assay. Five different concentrations of each fraction (A–D, see Figures 2 and 3)

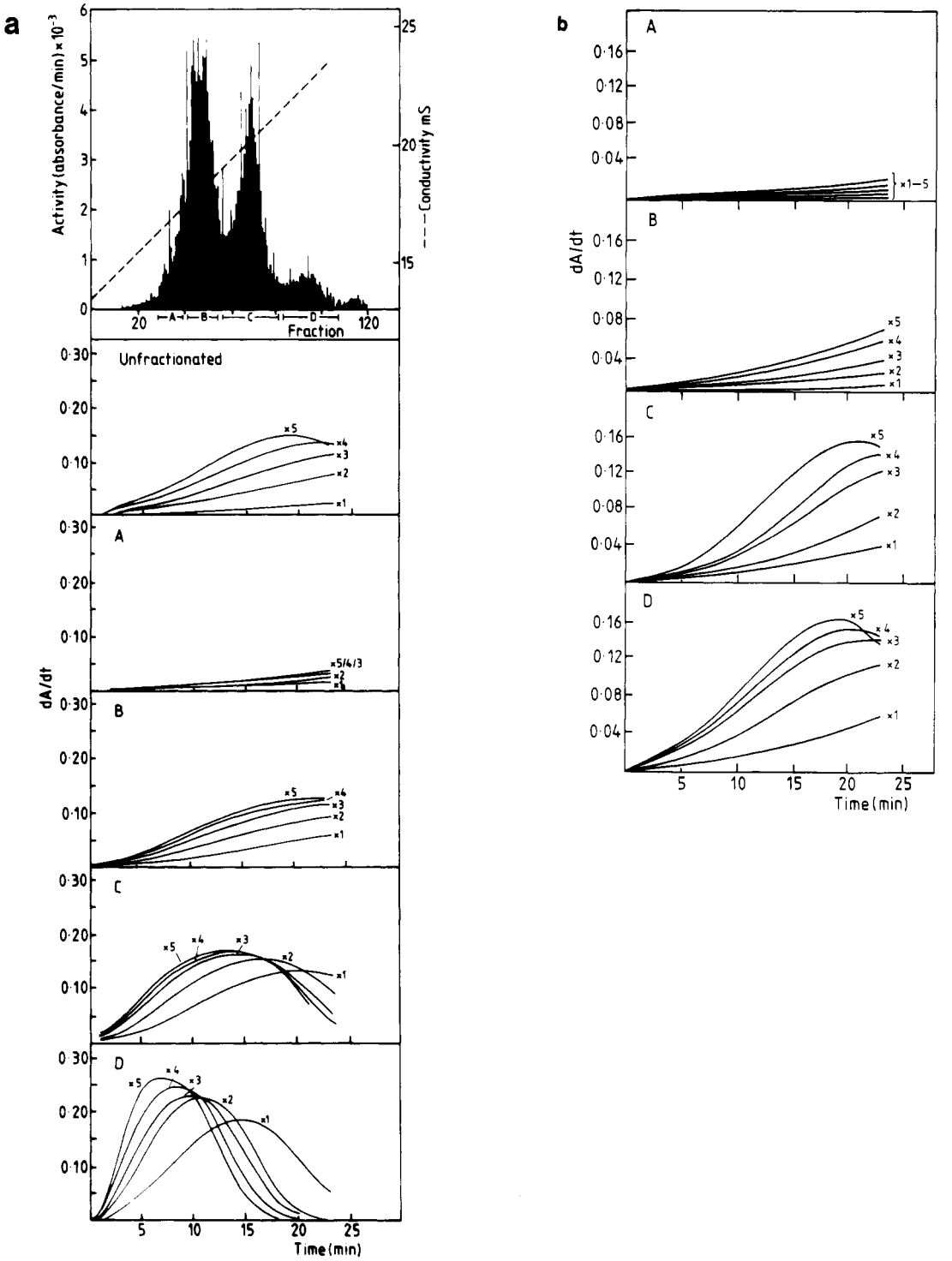

FIGURE 3: (Panel a) Murine rt-PA was fractionated by lysine–Sepharose chromatography. t-PA activity in each fraction was monitored by the direct amidolytic assay as described in Figure 2. Four peaks of activity were detected (A–D), and fractions were pooled as indicated. Fractions B and C correspond to type I and type II t-PA, respectively. The activity of each pool of murine rt-PA was measured by using S-2288, and an equivalent activity of each pool was then assayed in the stimulated indirect amidolytic assay (plasminogen 0.13 μM). The amounts of t-PA used were similar to those described for CHO rt-PA as described in the legend to Figure 2. A similar concentration of fibrin fragments was used in A–D. (Panel b) As in panel a, but with plasminogen concentration 1.3 μM.

and the unfractionated t-PA were assayed in the stimulated indirect amidolytic assay. Plasmin activity was followed by continually monitoring $dA(405\text{ nm})/dt$ with reaction time (Wittwer et al., 1989). The fractions of rt-PA clearly differ in this assay with respect to the level of plasmin produced within a given reaction time at constant fibrin levels. Also, the more retarded the fraction on lysine–Sepharose, the more rapid the production of plasmin. This was also the case for the different forms of t-PA derived from either a human colon fibroblast or the Bowes melanoma cell lines (Wittwer et al., 1989). In fact, the different fractions obtained by lysine–Sepharose of each t-PA appear to constitute a continuum of kinetically more active forms of t-PA [Figures 2 and 3 and Wittwer et al. (1989)].

Structural Analysis of the N-Linked Oligosaccharides Released from Unfractionated CHO and Murine rt-PA. An aliquot of the mixture of reduced ^{3}H-oligosaccharides isolated from either unfractionated CHO or murine rt-PA was sub-

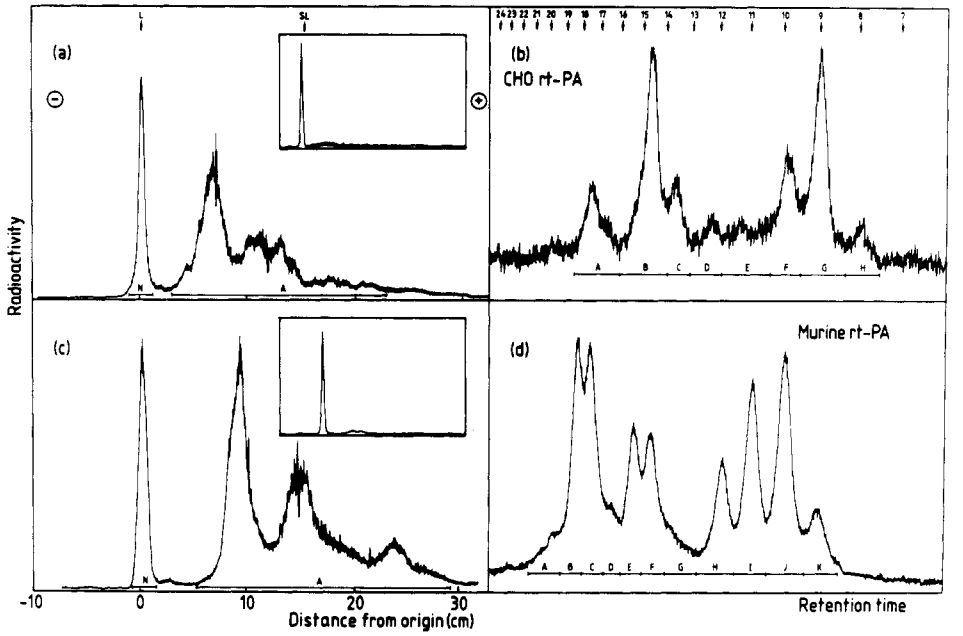

FIGURE 4: High-voltage radioelectrophoretograms (a, c) and Bio-Gel P-4 (~400 mesh) gel filtration chromatograms (b, d) of the oligosaccharides derived from CHO (a, b) and murine (c, d) rt-PA. Insets (a, c) are of the high-voltage radioelectrophoretograms of the oligosaccharides after exhaustive neuraminidase digestion. Following separation of the desialylated oligosaccharides by Bio-Gel P-4 (~400 mesh) gel permeation chromatography, individual fractions were pooled as indicated (b, d) for further analysis. Neutral (N) and acidic (A) oligosaccharides (a, c) were recovered by elution with water. L = lactitol; SL = 3′(6′)-sialyllactitol.

Table I: Relative Incidence of Neutral, Acidic, and Individual Desialylated Oligosaccharides Associated with Unfractionated, Type I, and Type II CHO rt-PA[a]

	CHO rt-PA (unfractionated)	CHO rt-PA type I	CHO rt-PA type II
neutral	55.2	41.4	69.9
acidic	44.8	58.6	30.1
A-1[b]	8.2	11.2	6.4
A-2	5.2	8.1	4.7
B	20.9	25.1	13.7
C	11.5	14.7	9.1
D	5.1	7.5	4.0
E-1	3.7	5.3	4.1
E-2	2.7	1.8	5.5
F	13.5	7.3	17.6
G	22.4	13.7	27.1
H	6.8	5.2	8.0
complex[c]	54.6	71.9	42.0
oligomannose[c]	45.4	28.0	58.2

[a] The relative incidence of neutral and acidic oligosaccharides was determined in each case as described under Materials and Methods. The relative incidence of individual desialylated oligosaccharides was determined by pooling fractions after Bio-Gel P-4 (~400 mesh) gel permeation chromatography of the total oligosaccharide pool (i.e., neutral and desialylated), as shown in Figure 4b. [b] See Figure 4 for structures. [c] The relative incidence of complex and oligomannose structures is, in each case, the sum of the relative incidence of fractions A-1 to E-1, and E-2 to H, respectively.

Table II: Relative Incidence of Neutral, Acidic, and Individual Desialylated Oligosaccharides Associated with Unfractionated, Type Ia, Type I, and Type II Murine rt-PA[a]

	murine rt-PA (unf)[b]	murine rt-PA type Ia	murine rt-PA type I	murine rt-PA type II
neutral	53.2	46.9	52.3	60.4
acidic	46.8	53.1	47.7	39.6
A[c]	2.2	3.3	2.4	2.5
B	11.2	11.8	10.4	9.6
C-1	10.1	9.3	9.1	8.9
C-2	1.9	2.7	1.9	1.8
D	1.7	2.7	2.3	2.1
E	8.6	13.4	10.5	8.1
F	8.9	10.7	8.6	6.3
G	3.5	4.4	4.8	4.5
H	8.2	9.2	10.4	9.1
I	17.8	13.4	15.4	16.5
J	20.1	15.9	19.2	21.3
K	5.6	3.2	5.0	9.2
complex[d]	48.1	58.3	50.0	43.8
oligomannose[d]	51.7	41.7	50.0	56.1

[a] The relative incidence of neutral and acidic oligosaccharides was determined in each case as described under Materials and Methods. The relative incidence of individual desialylated oligosaccharides was determined by pooling fractions after Bio-Gel P-4 (~400 mesh) gel permeation chromatography of the total oligosaccharide pool (i.e., neutral and desialylated), as shown in Figure 4d. [b] Unfractionated. [c] See Figure 5 for structures. [d] The relative incidence of complex and oligomannose structures is, in each case, the sum of the relative incidence of fractions A to G, and H to K, respectively.

jected to high-voltage paper electrophoresis (Figure 4a,c). The relative amount of neutral (N) and acidic (A) components was determined by recovery of radioactivity from paper (Tables I and II). Treatment prior to electrophoresis of the oligosaccharides released from unfractionated CHO rt-PA with either the Newcastle disease virus neuraminidase or with the neuraminidase from *Arthrobacter unreafaciens* caused conversion of all acidic oligosaccharides to neutral (Figure 4a, inset). It is therefore concluded that only nonreducing terminal sialic acid linked $\alpha 2 \rightarrow 3$ is responsible for the acidic nature of all CHO rt-PA associated acidic N-linked oligosaccharides. In the case of oligosaccharides released from unfractionated murine rt-PA, treatment prior to electrophoresis with Newcastle disease virus neuraminidase had no effect on the resulting radioelectrophoretogram (not shown), but prior treatment with the neuraminidase from *A. ureafaciens* caused conversion of all acidic oligosaccharides to neutral (Figure 4c, inset). It is therefore concluded that only nonreducing terminal sialic acid linked $\alpha 2 \rightarrow 6$ is responsible for the acidic nature of all murine rt-PA associated acidic N-linked oligosaccharides. The oligosaccharides obtained after neuraminidase digestion (i.e., neutral and desialylated acidic oligosaccharides combined) were separated on the basis of their effective hydrodynamic volume by Bio-Gel P-4 gel filtration chromatography (Figure

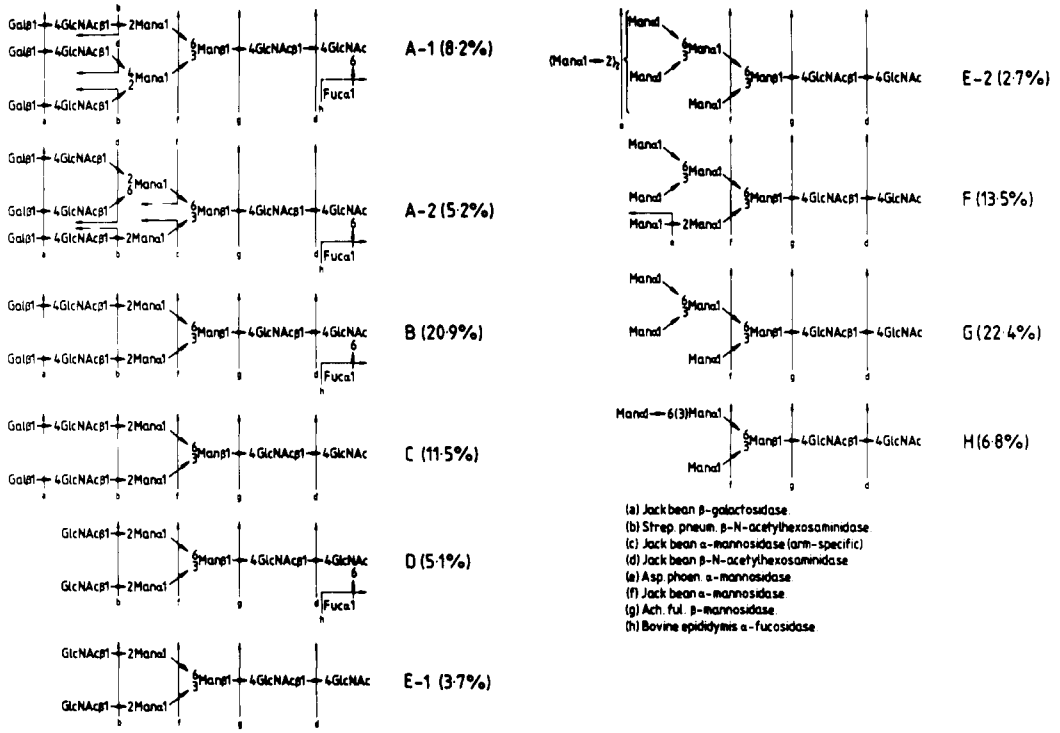

FIGURE 5: Structures of the neutral and desialylated N-linked oligosaccharides derived from unfractionated CHO rt-PA. Structural analysis was performed on individial oligosaccharide fractions (Figure 3b) by using a combination of sequential exoglycosidase digestion and methylation analysis. Changes in the hydrodynamic volume of oligosaccharide structures were effected by exoglycosidases when used in the following order: A-1, a-b-d-f-g-d-h; A-2, a-b-c-d-f-g-d-h; B, a-b-f-g-d-h; C, a-b-f-g-d; D, b-f-g-d-h; E-1, b-f-g-d; E-2, e-f-g-d; F, e-f-g-d; G, f-g-d; H, f-g-d. The deduced points of hydrolysis of each structure by individual exoglycosidases are indicated, together with the relative incidence of each structure in the total (neutral and desialylated) oligosaccharide pool. Where linkage positions could not be unequivocally established, all possible structures are shown. Since the total incidence of complex-type oligosaccharides carrying nonreducing terminal outer arm galactose (45.8%) is very similar to the total incidence of sialylated oligosaccharides (44.8%), it is concluded that essentially all these complex-type oligosaccharides of CHO rt-PA are at least monosialylated.

Table III: Methylation Analysis of the Desialylated Oligosaccharides Released from Unfractionated CHO rt-PA[a]

methylated monosaccharide	linkage	oligosaccharide fraction A	B	C	D	E	F	G	H
fucitol									
2,3,4-tri-*O*-methyl (1,5-di-*O*-acetyl)	t[b]	+[c]	+	−	+	−	−	−	−
galactitol									
2,3,4,6-tetra-*O*-methyl (1,5-di-*O*-acetyl)	t	+	+	+	−	−	−	−	−
mannitol									
2,3,4,6-tetra-*O*-methyl (1,5-di-*O*-acetyl)	t	−	−	−	−	+	+	+	+
3,4,6-tri-*O*-methyl (1,2,5-tri-*O*-acetyl)	2	+	+	+	+	+	+	−	−
2,3,4-tri-*O*-methyl (1,5,6-tri-*O*-acetyl)	6	−	−	−	−	−	−	−	+
3,6-di-*O*-methyl (1,2,4,5-tetra-*O*-acetyl)	2,4	+	−	−	−	−	−	−	−
3,4-di-*O*-methyl (1,2,5,6-tetra-*O*-acetyl)	2,6	+	−	−	−	−	−	−	−
2,4-di-*O*-methyl (1,3,5,6-tetra-*O*-acetyl)	3,6	+	+	+	+	+	+	+	+
2-(*N*-methylacetamido)-2-deoxyglucitol									
3,4,6-tri-*O*-methyl (1,5-di-*O*-acetyl)	t	−	−	−	(+)[d]	+	−	−	−
3,6-di-*O*-methyl (1,4,5-tri-*O*-acetyl)	4	+	+	+	+	+	+	+	+
1,3,5,6-tetra-*O*-methyl (4-mono-*O*-acetyl)	4OL	−	−	+	−	+	+	+	+
1,3,5-tri-*O*-methyl (4,6-di-*O*-acetyl)	4,6OL	+	+	−	+	−	−	−	−

[a] Following separation of the (dextran-free) total oligosaccharide pool (i.e., neutral and desialylated) released from unfractionated CHO rt-PA by Bio-Gel P-4 (~400 mesh) gel permeation chromatography, fractions were pooled as indicated in Figure 3b. Methylation analysis was then performed as described under Materials and Methods. [b] t = terminal. [c] (+) designates presence and (−) the absence of the partially methylated alditol acetate in the relevant fraction. [d] Only detectable in trace amount.

4b,d). Individual fractions were pooled as indicated, and the structures present within each fraction were determined by a combination of sequential exoglycosidase digestion and methylation analysis (of dextran-free material). Sensitivity of an oligosaccharide fraction to exoglycosidases (as judged by a decrease in effective hydrodynamic volume) when these are used in the order summarized in Figures 5 and 6 allows the determination of glycosyl residue sequence and anomeric configuration of individual glycosidic linkages and, in some cases, the positions of substitution of individual glycosyl residues [though methylation analysis (Tables III and IV) is used to define this rigorously]. The structures proposed to be associated with CHO and murine rt-PA (Figures 5 and 6) are consistent with the results of exoglycosidase digestion and methylation analysis.

Analysis of the N-Glycosylation of Various Forms of CHO and Murine rt-PA Obtained by Lysine–Sepharose Chromatography. CHO rt-PA and murine rt-PA were fractionated by lysine–Sepharose chromatography (Figures 2 and 3). The reduced oligosaccharides recovered from the two major forms (fractions B and C which correspond to type I and type II t-PA, respectively) were first analyzed with respect to their

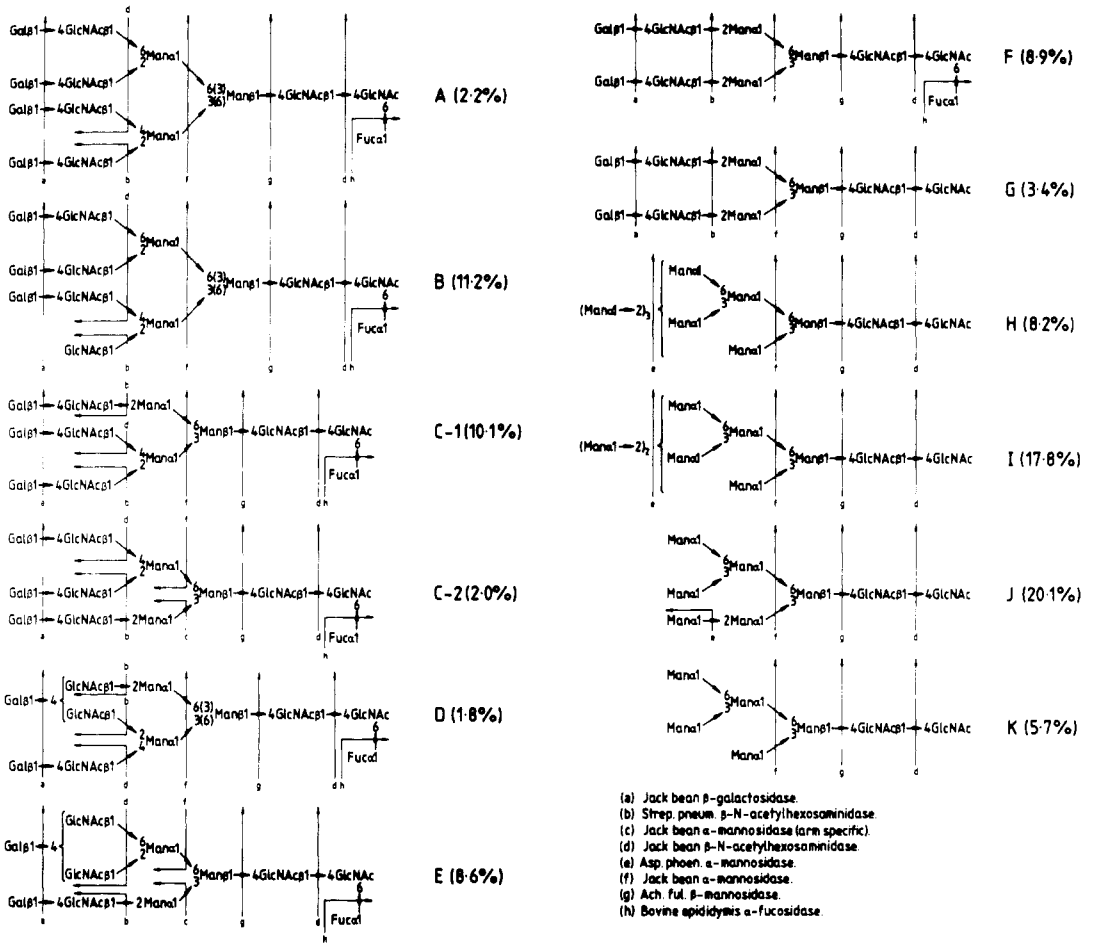

FIGURE 6: Structures of the neutral and desialylated N-linked oligosaccharides derived from unfractionated murine rt-PA. Structural analysis was performed on individual oligosaccharide fractions (Figure 3d) by using a combination of sequential exoglycosidase digestion and methylation analysis. Changes in the hydrodynamic volume of oligosaccharide structures were effected by exoglycosidases when used in the following order: A, a-b-d-f-g-d-h; B, b-a-d-f-g-d-h; C-1, a-b-d-f-g-d-h; C-2, a-b-c-d-f-g-d-h; D, a-b-d-f-g-d-h; E, a-b-c-d-f-g-d-h; F, a-b-f-g-d-h; G, a-b-f-g-d; H, e-f-g-d; I, e-f-g-d; J, e-f-g-d; K, f-g-d. The deduced points of hydrolysis of each structure by individual exoglycosidases are indicated, together with the relative incidence of each structure in the total (neutral and desialylated) oligosaccharide pool. Where linkage positions could not be unequivocally established, all possible structures are shown. Since the total incidence of complex-type oligosaccharides carrying nonreducing terminal outer arm galactose residues (48.2%) is very similar to the total incidence of sialylated oligosaccharides (46.8%), it is concluded that essentially all such oligosaccharides of murine C127 rt-PA are at least monosialylated.

Table IV: Methylation Analysis of the Desialylated Oligosaccharides Released from Unfractionated Murine rt-PA[a]

methylated monosaccharide	linkage	oligosaccharide fraction						
		A	B + C + D	E + F + G	H	I	J	K
fucitol								
2,3,4-tri-*O*-methyl (1,5-di-*O*-acetyl)	t[b]	+[c]	+	+	–	–	–	–
galactitol								
2,3,4,6-tetra-*O*-methyl (1,5-di-*O*-acetyl)	t	+	+	+	–	–	–	–
mannitol								
2,3,4,6-tetra-*O*-methyl (1,5-di-*O*-acetyl)	t	–	–	–	+	+	+	+
3,4,6-tri-*O*-methyl (1,2,5-tri-*O*-acetyl)	2	–	–	–	+	+	+	–
3,6-di-*O*-methyl (1,2,4,5-tetra-*O*-acetyl)	2,4	+	+	–	–	–	–	–
3,4-di-*O*-methyl (1,2,5,6-tetra-*O*-acetyl)	2,6	+	+	+	–	–	–	–
2,4-di-*O*-methyl (1,3,5,6-tetra-*O*-acetyl)	3,6	+	+	+	+	+	+	+
2-(*N*-methylacetamido)-2-deoxyglucitol								
3,4,6-tri-*O*-methyl (1,5-di-*O*-acetyl)	t	–	(+)[d]	+	–	–	–	–
3,6-di-*O*-methyl (1,4,5-tri-*O*-acetyl)	4	+	+	+	+	+	+	+
1,3,5,6-tetra-*O*-methyl (4-mono-*O*-acetyl)	4OL	–	–	+	+	+	+	+
1,3,5-tri-*O*-methyl (4,6-di-*O*-acetyl)	4,6OL	+	+	+	–	–	–	–

[a] Following separation of the (dextran-free) total oligosaccharide pool (i.e., neutral and desialylated) released from unfractionated murine rt-PA by Bio-Gel P-4 (~400 mesh) gel permeation chromatography, fractions were pooled as indicated in Figure 4d. The separation between fractions B, C, and D was not sufficient to allow these fractions to be recovered free of one another. These fractions were therefore pooled together for methylation analysis, and similarly for fractions E, F, and G. Methylation analysis was then performed as described under Materials and Methods. [b] t = terminal. [c] (+) designates presence and (–) the absence of the partially methylated alditol acetate in the relevant fraction. [d] Only detectable in trace amount.

relative content of neutral and sialylated oligosaccharides (as already described). Following incubation with neuraminidase, the total oligosaccharide pool (i.e., neutral and desialylated) was fractionated by Bio-Gel P-4 (~400 mesh) gel permeation chromatography, and oligosaccharide fractions were pooled as shown in Figure 4. In the case of fractions known (from

structural analysis of the oligosaccharides released from unfractionated CHO and murine rt-PA) to contain more than one oligosaccharide (i.e., A and E in CHO rt-PA, and C in murine rt-PA), the relative incidence of each individual structure was determined after sequential exoglycosidase analysis of that oligosaccharide fraction. The results are summarized in Tables I and II.

Discussion

The N-linked oligosaccharides found to be associated with the CHO rt-PA, and in particular, the occurrence of sialic acid only in the $\alpha 2 \rightarrow 3$ linkage, have been reported previously on other CHO-derived glycoproteins (Sasaki et al., 1987; Takeuchi et al., 1988; Oheda et al., 1988). The N-glycosylation of the murine rt-PA, while consistent with the known biosynthetic pathway for N-linked oligosaccharides (Schachter et al., 1983), is relatively unusual in that significant amounts of tri- and tetraantennary oligosaccharides which lack galactose on one outer arm are present. Whether these structures are generated after secretion of t-PA, for example, by the action of a β-galactosidase, or are true biosynthetic products is not clear. For both the CHO and murine C127 cell derived rt-PA, the incidence of sialylated structures is very similar to that of complex-type oligosaccharides possessing outer arm nonreducing terminal galactose residues (Tables I and II). This indicates that almost all such structures are at least monosialylated on the rt-PA polypeptide, unlike the t-PA isolated from the normal human colon fibroblast cell strain CDD-18Co (Parekh et al., 1989). Both the CHO rt-PA and murine rt-PA possess a significant number of oligosaccharides whose outer arms terminate in *N*-acetylglucosamine (8.8% and 10.4%, respectively). This was also found to be so for Bowes melanoma t-PA, but not human colon fibroblast t-PA (Parekh et al., 1989). With the exception of IgG, circulating serum glycoproteins possessing oligosaccharides whose outer arms terminate in *N*-acetylglucosamine are extremely rare (Rademacher et al., 1988a). On IgG these residues are buried within the peptide and are not accessible. This residue is known to be immunogenic when present on mycobacterial or bacteria cell surfaces (Rook et al., 1987; Chechik et al., 1987). Physiological consequences of such oligosaccharides on a circulating glycoprotein cannot be fully assessed at present but may include immune complex formation through interaction with naturally occurring anti-GlcNAc antibodies, possible activation of monocytes with consequent release of a variety of cytokines (Suzuki et al., 1985), and interaction with the various cellular receptors for outer arm nonreducing terminal *N*-acetylglucosamine (Rademacher et al., 1988a). The epitopes recognized by the IgE antibodies in the sera of patients with bee venom allergy also appear to be directed against the N-glycans of allergenic glycoproteins which carry nonreducing terminal *N*-acetylglucosamine and fucose (Marz et al., 1987).

As in the case of t-PA secreted by a Bowes melanoma and human colon fibroblast cell line (Wittwer et al., 1989), each of the various forms of CHO and murine rt-PA which are separable by lysine–Sepharose chromatography differ in their activity in the stimulated indirect amidolytic assay. This is not due to differences in fibrin-independent cleavage of plasminogen, since this is negligible for all the forms of t-PA (Wittwer et al., 1989) and rt-PA (data not shown). Rather, since t-PA attachment to fibrin involves, at least in part, its lysine-binding sites (Voskuilen et al., 1987), the more retarded fraction on lysine–Sepharose (type II) may have greater affinity for fibrin. Since type II t-PA lacks an N-linked oligosaccharide at Asn-184, the presence of this oligosaccharide may influence the ability of t-PA to bind fibrin through its lysine-binding sites and hence the fibrin-stimulated activity (down regulation in type I). Future efforts to probe the bioloical role of the t-PA oligosaccharide will involve a determination of relevant kinetic parameters in vitro using t-PA derived from different sources, and also an analysis of the N-glycosylation of native circulating human t-PA [see Rademacher and Dwek (1989) for a discussion of the possible physiological roles of slow and fast forms of t-PA]. A more detailed kinetic analysis has already been performed for type I and II t-PA from human colon fibroblast and Bowes melanoma (Wittwer et al., 1989).

Our studies to date indicate that distinct sets of t-PA glycoforms result from the expression of the same amino acid sequence by different cell lines. The sets differ with respect to the structure of certain oligosaccharides, the relative incidence of common oligosaccharides, and their fibrin-dependent plasminogenolytic activity. If generally true, this result implies that a recombinant glycoprotein expressed in a cell type in which it is not normally expressed would not constitute a "natural" product. It could differ from the natural product by possessing either novel oligosaccharides or novel *combinations* of oligosaccharide and polypeptide. In the latter case, therapeutic application could result in an immune response secondarily directed at the native molecule.

That "self" oligosaccharides may be immunogenic when covalently attached to "nonself" polypeptide is suggested by recent studies on the nature of the humoral immune response to viruses. The major part of this response is directed against the carbohydrate moieties of their surface glycoproteins (Alexander & Elder, 1984). This implies that "self" oligosaccharides expressed in the context of a viral polypeptide may be immunogenic. Consequently, great care must be taken to match as closely as possible not only the overall N-glycosylation of a recombinant product to that of the native one but also the N-glycosylation at each *site*. With the exception of human erythropoietin, there are very few examples in which the N-glycosylation of a recombinant human polypeptide has been compared to that of the native form (the N-glycosylation of native endothelial cell derived human t-PA has not yet been analyzed). In the case of erythropoietin, the recombinant form expressed in baby hamster kidney cells carried oligosaccharides not found on native urinary erythropoietin (Tsuda et al., 1988). However, the recombinant form expressed in CHO cells shared the same set of oligosaccharides as native erythropoietin, but in quite different relative amounts (Sasaki et al., 1987; Takeuchi et al., 1988). It should be noted that, in this latter case, the N-glycosylation at individual sites was not compared. Further, changes in the relative incidence of a constant set of polypeptide-associated N-linked oligosaccharides can have profound effects on the biological properties of that glycoprotein (Rademacher et al., 1988a).

In conclusion, the N-glycosylation of the human t-PA polypeptide when expressed in a normal human fibroblast cell strain, a Bowes melanoma cell line, CHO cells, and the murine C127 cell line has now been defined. The N-glycosylation of native endothelial cell derived human t-PA has not yet been analyzed, but our results indicate that the t-PA's described to date are N-glycosylated differently, supporting the view that N-glycosylation is cell-type-specific. Each t-PA analyzed was associated with certain unique oligosaccharide structures. For example, the "bisecting" GlcNAc is present only on melanoma-derived t-PA, $\alpha 2 \rightarrow 3$ linked sialic acid only on CHO cell derived rt-PA, hybrid-type oligosaccharides with nonreducing terminal outer arm galactose only on hcf-t-PA, and tetraantennary complex-type oligosaccharides with one nonreducing

terminal outer arm *N*-acetylglucosamine only on the murine rt-PA. Further, the relative incidence of common structures is also unique to each t-PA. Nevertheless, certain features of t-PA N-glycosylation appear to be conserved irrespective of the cell type in which the t-PA polypeptide is expressed. For example, the type II form invariably carries a higher incidence of oligomannose structures than does the type I, indicating that Asn-184 is associated mainly with oligosaccharides of the complex type in all cases. The incidence of oligomannose and complex structures on all type II forms is similar and is approximately 50%. This suggests that Asn-117 may be generally associated with oligomannose structures and Asn-448 with those of the complex type as shown for hcf- and m-t-PA (Parekh et al., 1989). It therefore appears likely that the t-PA polypeptide itself determines to a large extent the type of oligosaccharide that is to be carried at a given site, whereas the cell type determines the precise structure(s). These results are consistent with the wider literature, suggesting that different sites on a polypeptide control the class of oligosaccharide they carry with different stringency in each cell type (Rademacher et al., 1988a). Finally, in the case of t-PA, unlike rat thymocyte Thy-1 (Parekh et al., 1987), the N-glycosylation at one site does not appear to influence the N-glycosylation at another site. For example, for hcf- and m-t-PA the N-glycosylation at site-448 is unaffected by N-glycosylation at site-184, and despite Asn-117 carrying oligomannose-type oligosaccharides, complex-, hybrid-, and oligomannose-type oligosaccharides are all found at Asn-184.

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Castanospermine Inhibits the Function of the Low-Density Lipoprotein Receptor†

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ABSTRACT: Castanospermine, a plant alkaloid that inhibits the glycoprotein processing enzyme glucosidase I, has been used to inhibit N-linked oligosaccharide modification, resulting in the production of glycoproteins having $Glc_3Man_{7-9}(GlcNAc)_2$ oligosaccharides. This alkaloid caused a significant inhibition of LDL endocytosis in cultured primate smooth muscle cells and human skin fibroblasts. At an optimum concentration of 250 μg/mL, castanospermine caused a 40% decrease in cell surface receptor-mediated LDL binding at 4 °C, with no apparent change in affinity. Further, the inhibitor had no direct effect on LDL metabolism. This inhibition of LDL receptor expression and function occurred only when the drug was present during de novo receptor synthesis, i.e., during up-regulation. Although the number of cell surface LDL receptors was significantly reduced in the presence of castanospermine, the total number of receptors in the cell was only slightly reduced, indicating that castanospermine induced a redistribution rather than a reduction in the number of receptors. Similarly, subcellular fractionation studies confirmed that castanospermine treatment of fibroblasts results in an altered distribution of receptor activity compared with controls. These findings are consistent with the conclusion that the decrease in specific LDL binding to cells grown in the presence of castanospermine is due to intracellular redistribution of the LDL receptor so that more receptor remains in internal compartments as a result of a diminished rate of transport.

Many integral membrane proteins are glycoproteins that contain N-linked and O-linked oligosaccharides (Kornfeld & Kornfeld, 1985), and a number of these glycoproteins function as receptors for biologically important molecules such as transferrin (Omary & Trowbridge, 1981), insulin (Czech, 1982), acetylcholine (Prives & Bar-Sagi, 1983), and low-density lipoproteins (Brown & Goldstein, 1986). In each of these systems, the ligand–receptor complex is rapidly internalized through a process known as receptor-mediated endocytosis, which often, but not invariably, occurs in discrete clathrin-containing regions of the surface membrane called coated pits (Goldstein et al., 1976). At the present time, there is only preliminary and indirect evidence that suggests a role for the carbohydrate moiety in receptor recognition (Neufeld & Ashwell, 1981). However, much stronger evidence indicates that N-linked oligosaccharides are necessary for receptor function (Olden et al., 1978). Much of this evidence comes from the use of the antibiotic tunicamycin (Takatsu & Tamura, 1971), a compound which prevents N-linked glycosylation by inhibiting the formation of the first lipid-linked oligosaccharide intermediate, GlcNAc-PP-dolichol (Tkacz & Lampen, 1975). In the presence of tunicamycin, the insulin

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