

The glycosylation of Bowes melanoma tissue plasminogen activator: lectin mapping, reaction with anti-L2/HNK-1 antibodies and the presence of sulphated/glucuronic acid containing glycans

A. J. JAKES, G. OPDENAKKER, T. W. RADEMACHER, R. A. DWEK and S. E. ZAMZE*

Glycobiology Institute, Biochemistry Department, Oxford University, South Parks Road, Oxford OX1 3QU, U.K.

The glycosylation of tissue plasminogen activator (t-PA) obtained from the Bowes melanoma cell line was re-examined using methods of serial lectin affinity chromatography coupled with Bio-Gel P-4 gel filtration chromatography and exoglycosidase sequencing. This study clarified an earlier discrepancy in the literature and confirmed that the major complex N-linked glycans on Bowes t-PA that carry sialic acid as their sole charged group are bi-antennary, core fucosylated, with terminal N-

acetylgalactosamine residues. We also report the characterization of a series of related and previously unidentified sialylated glycans. Further we show that Bowes t-PA expresses glucuronic acid/sulphate containing N-linked glycans and is recognized by anti-carbohydrate L2/HNK-1 monoclonal antibodies. The presence on Bowes t-PA of glycans associated primarily with the nervous system is consistent with its expression in a cell line of neuroectodermal origin.

INTRODUCTION

Tissue plasminogen activator (t-PA) is a protease glycoprotein that converts the inactive proenzyme plasminogen into the active enzyme plasmin. It is a key component of the fibrinolytic system, where the degradation of fibrin by plasmin prevents the accumulation of fibrin thrombi in the vasculature [1].

As well as a role in vascular fibrinolysis, plasminogen activators (PAs) are expressed in many tissues and are believed to function in proteolysis of the extracellular matrix during development and regeneration [2]. The brain and neuroendocrine tissues in particular have high levels of t-PA activity [3] and there is increasing evidence to suggest that PAs in the nervous system play a role in regulating cell migration and neurite outgrowth during development [4,5]. For example, PA expression has been shown to coincide with extensive cell migration and proliferation and tissue remodelling in the developing central nervous system [6,7]; there is localized expression of PA at the growth cone of cultured peripheral neurons [8]; and neuronal cells express surface receptors for t-PA [5]. Production of t-PA by the neuronal cell line PC12 influences neurite extension and cell migration in a complex extracellular matrix *in vitro* [9,10].

Extracellular proteolysis catalysed by neuronal t-PA has also been shown to occur in the adult mouse brain, suggesting that t-PA also contributes to adult central nervous system physiology [11], such as long-term potentiation [12].

t-PA has four potential N-glycosylation sites, only three of which, at Asn-117, Asn-184 and Asn-448, are occupied. It occurs naturally as two major variants, designated type I and type II, differing in their degree of site occupancy. In type I t-PA, glycans are present at all three sites, whereas in type II t-PA glycans are present only at sites 117 and 448 [13]. t-PA has been isolated from several sources such as human placenta [14], the Bowes melanoma cell line [15,16], and transfected CHO [17] and mouse epithelial (C127) cells [18]. The glycosylation of t-PA at sites 184 and 448 has been found to vary considerably between cell types,

whereas site 117 invariably contains mainly oligomannosidic glycans. t-PA also contains an O-linked fucose residue attached to Thr-61 in the epidermal growth factor domain [19].

The Bowes melanoma cell line [15] is of neuroectodermal origin. The glycosylation of this molecule has been studied in a number of laboratories [20–22] and shown to be quite distinctive compared with t-PA from other sources. However, discrepancies have occurred in the reported structures.

Parekh et al. [21] reported the presence of a series of novel truncated bi-, tri-, tetra- and penta-antennary structures in which each antenna comprised a single GlcNAc residue. In addition, sulphated glycans were also reported to be a major component, constituting up to 30% of the total glycan pool. Chan et al. [22], using fast atom bombardment mass spectrometry to analyse the N-linked glycans, reported that the major complex structure was an unusual mono-sialylated bi-antennary glycan containing terminal GalNAc instead of Gal residues. N-linked oligosaccharides containing the GalNAc β 1,4GlcNAc structural unit were first described for the pituitary glycoprotein hormones [23,24], in which the GalNAc residue is 4-O-sulphated. The same or a very similar structural unit, in which GalNAc is substituted with either α (2,3)- or α (2,6)-linked sialic acid or is unsubstituted, has since been found on a growing number of glycoproteins (reviewed in [25]).

To clarify the earlier work, and because of the biological interest in neurally derived t-PA, we re-examined some of the structures of interest on Bowes t-PA using techniques of serial lectin affinity chromatography coupled with Bio-Gel P-4 chromatography and exoglycosidase sequencing. In this paper we report that the only neutral glycans present in any significant quantity on Bowes t-PA are oligomannosidic and that the complex glycans containing sialic acid as their sole charged species are a series of sialylated GalNAc-containing structures, the major component of which is in agreement with that reported by Chan et al. [22]. We also provide new evidence for the presence of significant amounts of sulphated and glucuronic acid-containing glycans

Abbreviations used: AAL, *Aleuria aurantia* lectin; GC-MS, gas chromatography-mass spectrometry; gu, glucose units; HVE, high-voltage paper electrophoresis; MALDI-MS, matrix-assisted laser desorption-mass spectrometry; PA, plasminogen activator; PNGase F, peptide-N-glycosidase F; RCA, *Ricinus communis* agglutinin; SLAC/GFC, serial lectin affinity chromatography/gel filtration chromatography; TMS, trimethylsilyl; t-PA, tissue plasminogen activator; WFA, *Wisteria floribunda* agglutinin.

* To whom correspondence should be addressed.

(up to 40 % of total glycans) and show that L2/HNK-1 monoclonal antibodies, which recognize a carbohydrate epitope based on a terminal 3-sulphated glucuronic acid moiety [26,27], react with Bowes t-PA. This epitope is prevalent in neural tissues, where it is expressed on glycoproteins, proteoglycans and glycolipids in both the peripheral and central nervous systems [27–32]. Its identification on Bowes t-PA is therefore consistent with the neuroectodermal origin of this cell line. Outside the nervous system, the L2/HNK-1 epitope is known to be present only on a subset of human lymphocytes including natural killer cells [33].

MATERIALS AND METHODS

Materials

t-PA from the Bowes melanoma cell line was purchased from American Diagnostica (Greenwich, CT, U.S.A.). *Aleuria aurantia* lectin (AAL) was purchased from Boehringer Mannheim U.K. (Lewes, E. Sussex, U.K.) and coupled to Affigel-10 (Bio-Rad Labs Ltd., Watford, U.K.) in accordance with the manufacturer's instructions. *Ricinus communis* agglutinin (RCA)-120-agarose and *Wisteria floribunda* agglutinin (WFA)-agarose were purchased from Vector Labs (Peterborough, U.K.). Concanavalin A-Sepharose was purchased from Sigma Chemical Co. (Poole, Dorset, U.K.).

Exoglycosidases were either prepared as previously described [21] or purchased from Oxford GlycoSystems (Abingdon, Oxon., U.K.).

Anti-L2/HNK-1 specific rat IgG monoclonal antibodies, 336 and 412 [30,34], and glycoproteins L1 and tenascin-R (janusin) were obtained from Professor M. Schachner.

Release and radiolabelling of N-linked oligosaccharides, high-voltage paper electrophoresis and Bio-Gel P-4 gel filtration chromatography

The release of N-linked oligosaccharides by hydrazinolysis followed by their purification and radiolabelling by reduction with NaBH_4 , and analysis by high-voltage paper electrophoresis (HVE) and Bio-Gel P-4 gel filtration chromatography were all performed as previously described [21,35,36].

Desialylation by mild acid hydrolysis

Oligosaccharides were desialylated by heating at 100 °C for 30 min in 2 M acetic acid. Acid was removed by evaporation under reduced pressure.

Lectin affinity chromatography

Oligosaccharides previously neutralized by digestion with *Arthrobacter ureafaciens* neuraminidase were analysed by serial lectin affinity chromatography. Chromatography on AAL, WFA and RCA-120 was performed on 5 cm × 0.5 cm columns (1 ml bed volume). In all cases samples were applied in 50 µl of equilibration buffer and fractions (0.25 ml) were collected and assayed for radioactivity. Pooled radioactive peaks (unbound, retarded or bound) were desalted by passage through a column either of Dowex AG50 x 12 (H^+) or of the same resin layered over Dowex AG3 x 4A (OH^-) and analysed further by Bio-Gel P-4 chromatography and exoglycosidase digestions.

Chromatography on AAL (specificity for Fuc α 1,6-linked to the terminal reducing GlcNAc residue) was performed in 0.1 M sodium acetate, pH 6.0, at a flow rate of 50 µl/min [37]. Oligosaccharides (unbound) were eluted with buffer alone (5 ml), followed by elution of bound oligosaccharides with buffer containing 1 mM L-Fuc (3 ml). Chromatography on WFA (speci-

ficity for terminal GalNAc) [38,39] and RCA-120 (specificity for terminal Gal) [39] was performed in PBS (0.015 M sodium phosphate, 0.15 M sodium chloride, pH 7.2) at a flow rate of 50 µl/min. Unbound oligosaccharides were eluted with buffer (7.5 ml). Bound oligosaccharides were eluted from WFA in buffer containing 5 mM *N*-acetylgalactosamine (4 ml) and from RCA in buffer containing 10 mM lactose (4 ml). Fractions of 250 µl were collected.

Chromatography on concanavalin A (0.6 ml) was performed in 0.01 M Tris-buffered saline, pH 8.0 (0.01 M Tris, 0.15 M NaCl, 1 mM CaCl_2 , 1 mM MgCl_2) at 0.5 ml/min based on previously described methods [40]. Unbound oligosaccharides were eluted in buffer (6 ml), bound glycans were eluted with buffer containing 10 mM α -methyl-D-glucoside (6 ml) followed by elution with buffer containing 100 mM α -methyl-D-mannoside (6 ml) and finally with 0.1 M HCl. Fractions of 0.6 ml were collected.

Desulphation by mild methanolysis

Oligosaccharides were desulphated by incubation in 500 µl of dry 50 mM methanolic HCl at 25 °C for 18 h. The reaction was stopped by evaporation to dryness under reduced pressure and residual acid was removed by repeated evaporation from water. Oligosaccharides were then *N*-acetylated by incubation in 500 µl of saturated sodium bicarbonate and 20 µl of acetic anhydride for 10 min at 4 °C, followed by the addition of a further 20 µl of acetic anhydride and incubation at 23 °C for 50 min. Samples were desalted by passage through a column of Dowex AG50 x 12 (H^+) before further analysis. Carboxy groups, which become methylated during the above procedure, were subsequently de-esterified by treatment with 50 mM sodium hydroxide at 50 °C for 2 h. Samples were desalted as described above.

Exoglycosidase digestions

The digestion of radiolabelled glycans with exoglycosidases of defined specificity was performed as previously described [21]. The products of digestion were desalted by passage through mixed ion-exchange resins as previously described [21] and analysed by Bio-Gel P-4 chromatography on a GlycoMap 1000 (Oxford GlycoSystems Ltd.) column. Digestion with *Vibrio cholerae* neuraminidase (Boehringer-Mannheim U.K.) was performed at 1 unit/ml in 50 mM sodium acetate, 0.15 M NaCl, 9 mM CaCl_2 , pH 5.5, in a volume of 20 µl at 37 °C for 16 h.

Fractionation of acidic glycans on Dowex AG3 x 4A anion-exchange resin

Glycans that retained an anionic charge after exhaustive desialylation were fractionated into sulphated and non-sulphated pools by passage through a 200 µl column of Dowex AG3 x 4A (acetate form). Unbound glycans (non-sulphated) were eluted with 5 × 200 µl of 4 M acetic acid. Acetic acid was removed by evaporation under reduced pressure. Bound glycans (sulphated) were eluted with 1 ml of 0.5 M sodium hydroxide and desalted by passage through Dowex AG50 x 12 (H^+).

Anion-exchange HPLC

Anion-exchange HPLC was performed on either (a) a MicroPak AX-5 column (25 cm × 0.46 cm) (Varian) in ammonium acetate, pH 4.5, at a flow rate of 0.5 ml/min, or (b) a Vydac 301HP575 weak anion-exchange column (0.7 cm × 5 cm) (Hichrom) in ammonium formate at pH 9.0, flow rate 1 ml/min [41]. The following linear stepwise gradients were used: for column (a), 5 mM acetate, held for 5 min, followed by increases to 125 mM

over 30 min, 250 mM over 5 min, held for 10 min, and then increased to 500 mM over 5 min and held for 5 min; for column (b), an increase from 0 to 25 mM formate over 12 min, followed by increases to 105 mM over 13 min, 400 mM over 25 min, 500 mM over 5 min, then held for 5 min. In all cases fractions of 0.5 ml were collected and assayed for radioactivity.

ELISA

The wells of microtitre plates (Polystyrene, NUNC) were coated with test glycoproteins (50 μ l) at concentrations of 5, 20 and 50 μ g/ml by incubation in sodium carbonate, pH 9.6, for 18 h at 4 °C, followed by washing three times with PBS, pH 7.2, containing 0.05 mg/ml Tween-20 (PBS/Tween-20).

For direct binding assays the coated wells were incubated in triplicate with 50 μ l aliquots of anti-L2/HNK-1 monoclonal antibodies 336 (1:1000 dilution) and 412 (1:5000 dilution) in PBS/Tween-20/BSA for 2 h at 25 °C.

For competitive ELISA, monoclonal antibody 336 (1:1000 dilution) was pre-incubated with different concentrations of the L2/HNK-1 positive glycoprotein, tenascin-R, for 90 min at 23 °C before being transferred in triplicate to microtitre plate wells coated with t-PA (5 μ g/ml), followed by incubation for a further 30 min at 23 °C.

In each case, the plates were then washed three times with PBS/Tween-20 and bound antibodies detected by incubation with anti-rat IgG conjugated with horseradish peroxidase (1:500 dilution in PBS/Tween-20) for 2 h at 23 °C. The plates were washed as before and the assay developed by the addition of 100 μ l of 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) (2 mg/ml) in 0.05 M citrate/phosphate buffer, pH 5.0, containing 0.05 % (v/v) hydrogen peroxide (100 vol.). The reaction was stopped by the addition of 100 μ l of sodium fluoride (24 mg/ml in distilled water. Absorbance was measured at 650–490 nm.

PAGE and immunoblotting (Western blotting)

Proteins were resolved by SDS/PAGE by the method of Laemmli [42] on 10 % (w/v) polyacrylamide slab gels. The gels were either stained with Coomassie Blue or the proteins were electrophoretically transferred onto a nitrocellulose sheet at 36 V for 18 h. For immunodetection, blots were incubated with shaking in PBS, pH 7.2, containing 5 % (w/v) skimmed milk powder for 4 h at 23 °C, followed by incubation with either monoclonal antibody 336 or 412 (1:500 and 1:1000 dilutions respectively) in the same buffer for 18 h at 4 °C. After washing, the blots were developed with 3,3'-diaminobenzidine tetrahydrochloride (100 μ M) in PBS containing 0.05 % (v/v) hydrogen peroxide (100 vol.).

Monosaccharide compositional analysis

Monosaccharide composition was determined by GC-MS of the 1-*O*-methyl trimethylsilyl (TMS) derivatives. Monosaccharides were released from the intact glycoprotein (50 μ g) by methanolysis (dry 0.5 M methanolic HCl at 70 °C for 16 h) followed by *N*-acetylation as described previously [43]. The 1-*O*-methyl glycosides were derivatized by reaction with *N,O*-bis-(trimethylsilyl) trifluoroacetamide/acetonitrile (1:1 by vol.) for 10 min at 60 °C. *scyllo*-Inositol (2 nmol) was added as an internal standard.

GC-MS was performed with a Fisons VG Autospec QFPD mass spectrometer interfaced to a Hewlett Packard series 11 GC with splitless injection. Spectra were recorded at 70 eV. Separations were carried out on a 25 m $\times$ 0.25 mm fused-silica OV-1 capillary column with a temperature programme of 80 °C, held

for 3 min, followed by a linear increase to 180 °C at 20 °C/min and a linear increase to 250 °C at 5 °C/min.

Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS)

MALDI-MS was performed with a Finnigan-MAT LaserMat with a pulsed nitrogen laser at 337 nm. Glycans (approx. 0.5 nmol) were analysed with 2,5-dihydroxybenzoic acid as the matrix.

Sulphate labelling of Bowes melanoma t-PA

Confluent cultures (approx. 10^8 cells) of mycoplasma-free Bowes melanoma cells were washed twice with sulphate-free medium and then incubated for 16 h in 20 ml of sulphate-free medium containing 10 % (w/v) dialysed serum and 25 μ Ci/ml [35 S]sulphate. During the incubation the cell cultures were either stimulated with 100 ng/ml phorbol 12-myristate 13-acetate to enhance t-PA production [44] or left untreated.

The culture media were concentrated by ultracentrifugation (Millipore Ultrafree, cut-off 10 kDa) and incorporation of 35 S into macromolecular material was measured by trichloroacetic acid precipitation. Incorporation was more than 87 % for all samples analysed. t-PA was purified from the concentrate by adsorption to a t-PA-specific antibody [45] and precipitation with Protein A-coated staphylococci (Pansorbin cells; Calbiochem), followed by extensive washing.

Peptide-N-glycosidase F (PNGase F) digestion

t-PA (20 μ g) was denatured by heating at 100 °C for 2 min in 10 μ l of phosphate/EDTA, pH 7.5, containing 0.5 % (w/v) SDS and 5 % (v/v) 2-mercaptoethanol followed by the addition of 2.5 μ l of 10 % (w/v) Nonidet P40 (final concentration 1.25 %), and 2 units of PNGase F (7.5 μ l). Incubation was carried out for 18 h at 37 °C.

Autoradiography

35 S-labelled t-PA samples (before and after PNGase F treatment) were separated by SDS/PAGE on a 15 % polyacrylamide slab gel. Autoradiography was carried out for 3 days with intensifying screens at –70 °C on Agfa Curix RP2 film.

RESULTS

HVE and Bio-Gel P-4 chromatography

HVE of the radiolabelled glycans revealed a neutral component (N, retained at the origin) and a series of acidic peaks, Ac_{1–4} (Figure 1a). The total glycan pool (neutral and acidic) was separated by Bio-Gel P-4 chromatography into an acidic fraction (eluting in the void volume) and a neutral fraction. The neutral glycans were resolved into a series of components (Figure 2). These were confirmed, by exoglycosidase sequencing with *Aspergillus saitoi* α (1,2)-specific mannosidase and jack bean α -mannosidase (non-specific), to consist mainly of the oligo-mannosidic glycans Man_{7–8}GlcNAc₂ (fractions c–e respectively) together with smaller amounts of Man₈GlcNAc₂ and Man₄GlcNAc₂ (fractions b and f). Trace amounts of neutral complex glycans that were resistant to α -mannosidase digestion were contained in fraction a.

A fraction of the the acidic glycans pooled from P-4 were re-chromatographed by HVE (Figure 1b), confirming that the acidic structures shown in Figure 1(a) had been recovered in the same molar proportions. The remainder of the acidic glycans

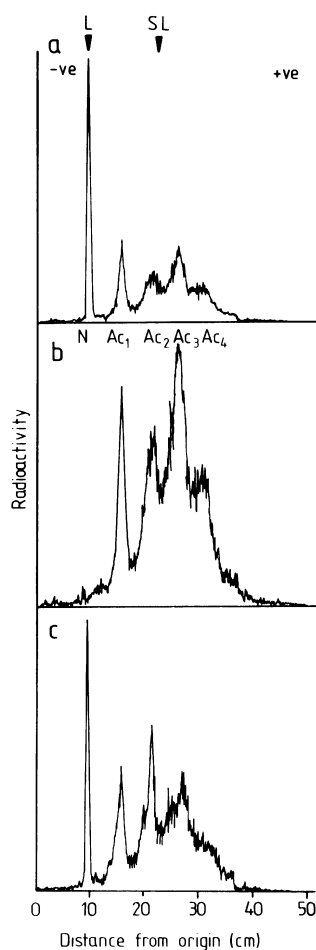


Figure 1 HVE of Bowes melanoma t-PA radiolabelled oligosaccharides

(a) Total glycans; (b) acidic glycan pool after separation on Bio-Gel P-4; (c) acidic glycans after exhaustive neuraminidase digestion. The oligosaccharides were subjected to HVE (80 V/cm) in pyridine/acetic acid/water (3:1:387, by vol.), pH 5.4. The arrows indicate the positions of [^3H]lactitol (L) and 6'(3')-sialyl-[^3H]lactitol (SL).

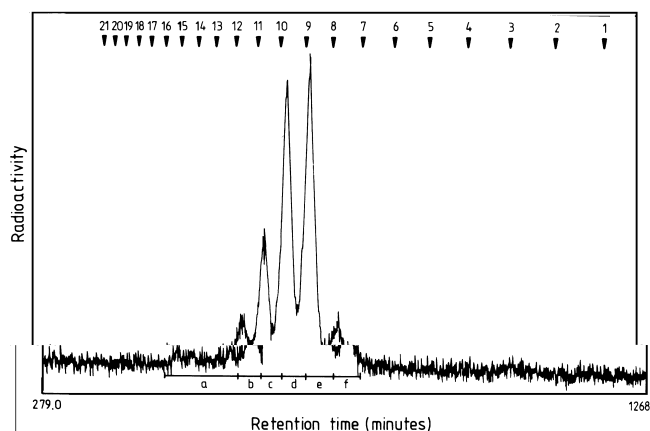


Figure 2 Bio-Gel P-4 chromatogram of the naturally occurring neutral oligosaccharides from Bowes melanoma t-PA

Fractions a–f contain the following percentages of total glycans present in Bowes t-PA: a, 1.4%; b, 3%; c, 7.3%; d, 14.5%; e, 18.5%; f, 3.2%. Numbers shown at the top indicate the elution positions of glucose oligomers from a dextran hydrolysate.

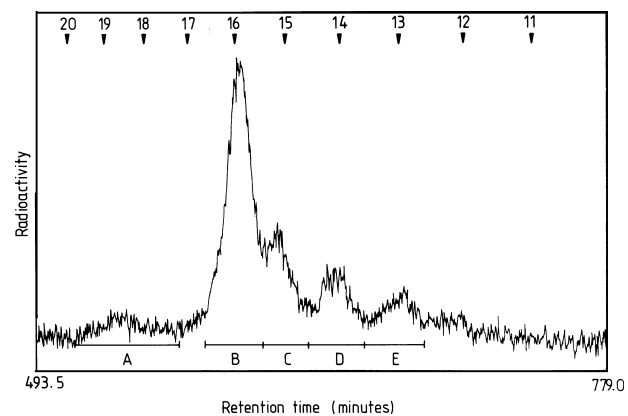


Figure 3 Bio-Gel P-4 chromatogram of the neutral desialylated oligosaccharides from Bowes melanoma t-PA

Pools A–E contain the complex-type oligosaccharides shown in Tables 1 and 2 and in Figure 4.

Separation by *Aleuria aurantia* Lectin (AAL) Affinity Chromatography
Specificity for reducing terminal Fuc (α 1-6) GlcNAc



Bio-Gel P-4 Gel Filtration Chromatography



Separation by Ricin (RCA_{120}) Lectin Affinity Chromatography
Retains outer-arm galactosylated oligosaccharides
(mono-galactosylated are retarded, di-galactosylated are retained -eluted with lactose)



Bio-Gel P-4 Gel Filtration Chromatography



Analysis by Concanavalin (Con-A) Lectin Affinity Chromatography
Retains oligosaccharides with at least two outer-arm mannose residues that are either unsubstituted or only at C-2 by another sugar:
tri- or tetra-antennary complex oligosaccharides - unbound;
di-antennary complex - retained, eluted with 10mM methyl- α -D-glucoside (a);
hybrid & certain high mannose oligosaccharides - retained, eluted with 100mM methyl- α -D-mannoside (b).



Analysis by *Wisteria floribunda* agglutinin (WFA) (Japanese Wisteria)
Lectin Affinity Chromatography
Retains outer-arm *N*-acetylgalactosamine containing oligosaccharides

Scheme 1 Strategy for analysis by SLAC/Bio-Gel P-4 gel filtration chromatography of the desialylated neutral glycans from Bowes melanoma t-PA

were digested with *Arthrobacter ureafaciens* neuraminidase. The resulting HVE profile (Figure 1c) shows that complete neutralization was achieved for only a fraction of the acidic glycans. Shifts in the acidic part of the chromatogram compared with the profile of the original material, i.e. a relative decrease in the amount of material in the Ac_{3-4} region, indicated that some of the charge on these acidic structures was due to sialic acid. Further treatment of the acidic glycan fraction either by digestion with *Vibrio cholerae* neuraminidase (broader specificity than *Arth-*

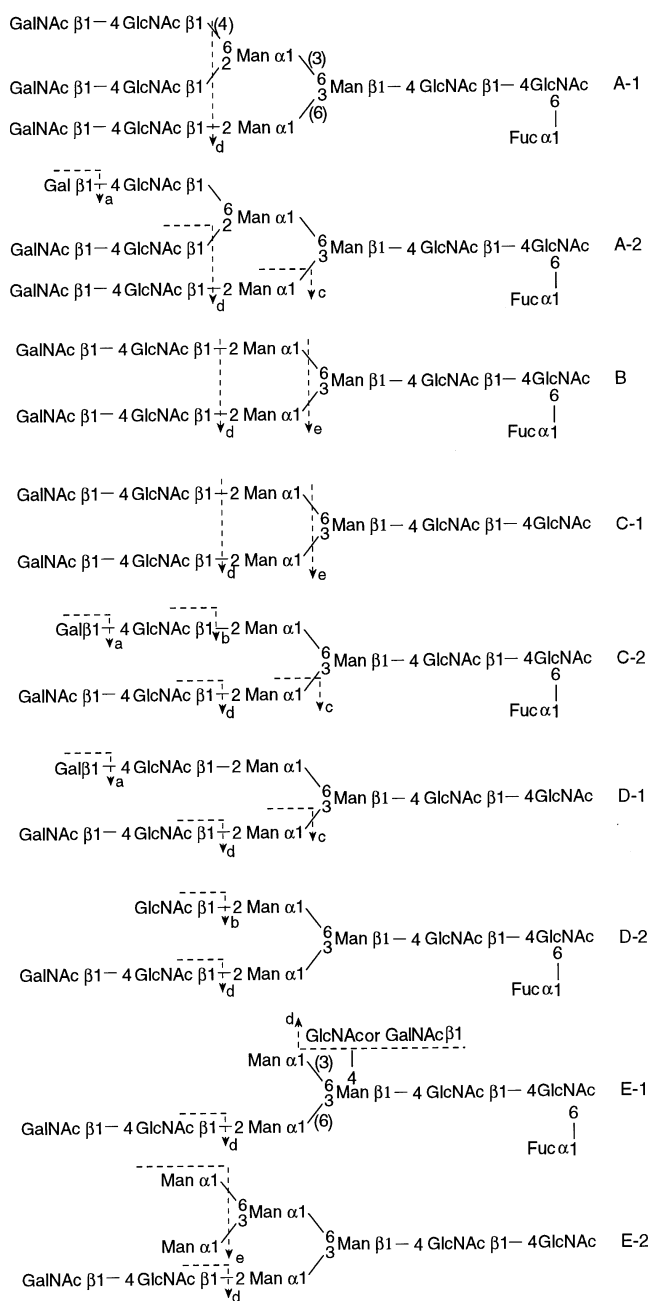


Figure 4 Structures of the major desialylated N-linked oligosaccharides from Bowes melanoma t-PA and their pattern of sequential exoglycosidase digestion

The enzymes used were: a, *Diplococcus pneumoniae* β -galactosidase; b, *Diplococcus pneumoniae* β -N-acetylhexosaminidase; c, jack bean α -mannosidase under arm-specific conditions [i.e. where R-Man α 1-6(Man α 1-3)Man β 1-4GlcNAc β 1-4GlcNAc but not R-Man α 1-3(Man α 1-6)Man β 1-4GlcNAc β 1-4GlcNAc is susceptible and where R is not H or Man]; d, jack bean β -N-acetylhexosaminidase; e, jack bean α -mannosidase at 25 units/ml. Exoglycosidases were used in the following order (negative digest in brackets): A-1, (b)-d; A-2, (b)-d-c-a; B, (b)-d-e; C-1, (b)-d-e; C-2, (b)-d-c-a-b; D-1, (b)-d-c-a; D-2, b-(c)-d; E-1, (c)-(b)-d; E-2, (b)-e-d. All enzyme digests were performed under conditions previously described [21]. Digestion with *Diplococcus* β -hexosaminidase was carried out at 0.1 unit/ml, under which no GalNAc β 1-4 linkages were cleaved. For digestion with jack bean α -mannosidase under arm-specific conditions, digestions were performed at an enzyme concentration of 10 units/ml in 15 μ l of 0.1 M sodium acetate, pH 5.0, for 18 h at 37 °C.

robacter neuraminidase) or by mild acid hydrolysis failed to cause any further neutralization, indicating that the charged groups were not due to unusual sialic acid substitutions.

Table 1 Analysis by SLAC/GFC of the de-sialylated neutral glycan pool retained by *Aleuria aurantia* lectin

The size of oligosaccharides given in glucose units (gu) refers to their hydrodynamic volume relative to an internal standard of glucose oligomers. ConA, concanavalin A. Symbols used in structures: ★, fucose; ▲, galactose; ×, N-acetylglucosamine; ■, N-acetylglucosamine; ●, mannose.

Lectin binding†				Gel filtration glucose units	Percentage sialylated pool	Proposed structure	Structure confirmed by enzyme digest ‡
Aleuria aurantia	Ricin	Con-A ◆	Wisteria floribunda				
+	-	-	+	18.7	2.7		✓(A-1)
+	-	†(a)	+	16	49.8		✓(B)
+	-	†(a)Ret. (b)	+	14.1	12.5		✓(D-2)
+	-	-	+	13.1	5.3		✓(E-1)
+	-	†(a)	+	13.1	2.0		×
+	Ret.	-	+	18.2	1.6		✓(A-2)
+	Ret.	†(a)	+	15.2	12.6		✓(C-2)
+	Ret.	†(a)	+/-	14.1	1.1		×
+	Ret.	†(a)	-	13.2	0.8		×
+	+	-	+/-	18.1 & 17.6	1.1		×
+	+	-	-	15.1	2.2		×

† Lectin: +, bound; -, unbound; Ret., retarded.

◆ (a) Eluted with 10 mM α -methylglucoside; (b) eluted with 100 mM α -methylmannoside.

‡ Structure (in parentheses) as given in Figure 4.

The desialylated glycans were again separated by Bio-Gel P-4 chromatography into acidic and neutral pools. The P-4 profile of the desialylated neutral glycans (Figure 3) revealed a major component (B) eluting at 16.0 glucose units (gu) and further components eluting at 18.7 gu (A) and from 15.2 to 13.0 gu (C-E).

The neutral glycans (naturally occurring, mainly oligomannose) accounted for 47–50 % of the total structures present, the complex glycans containing sialic acid as their sole charged group accounted for 13–15 %, and the remaining acidic glycans, containing charged groups in addition to sialic acid, accounted for 37–38 % of total glycans.

Serial lectin affinity chromatography/gel filtration chromatography (SLAC/GFC)

The neutral desialylated complex glycans were analysed in detail by SLAC/GFC, following the procedure shown in Scheme 1. The structures of the more abundant oligosaccharides were confirmed by sequential exoglycosidase digestions as shown in Figure 4. Assignment of the number of glycosyl residues removed

Table 2 Analysis by SLAC/GFC of the de-sialylated neutral glycan pool not retained by *Aleuria aurantia* lectin

The size of oligosaccharides given in glucose units (gu) refers to their hydrodynamic volume relative to an internal standard of glucose oligomers. ConA, concanavalin A. Symbols used in structures: ▲, galactose; ✕, *N*-acetylgalactosamine; ■, *N*-acetylglucosamine; ●, mannose.

Lectin binding†				Gel filtration glucose units	Percentage sialylated pool	Proposed structure	Structure confirmed by enzyme digest‡
<i>Aleuria aurantia</i>	Ricin	Con-A◆	Wisteria floribunda				
-	-	+(a)	+	15	3.7		✓(C-1)
-	-	+(b)	+	13	2.9		✓(E-2)
-	Ret.	+(a)	+	14.4	1.0		✓(D-1)
-	+	-	-	14	0.3		✕
-	+	+(a)	-	13.4	0.4		✕

† Lectin: +, bound; -, unbound; Ret., retarded.
◆ (a) Eluted with 10 mM α -methylglucoside; (b) eluted with 100 mM α -methylmannoside.
‡ Structure (in parentheses) as given in Figure 4.

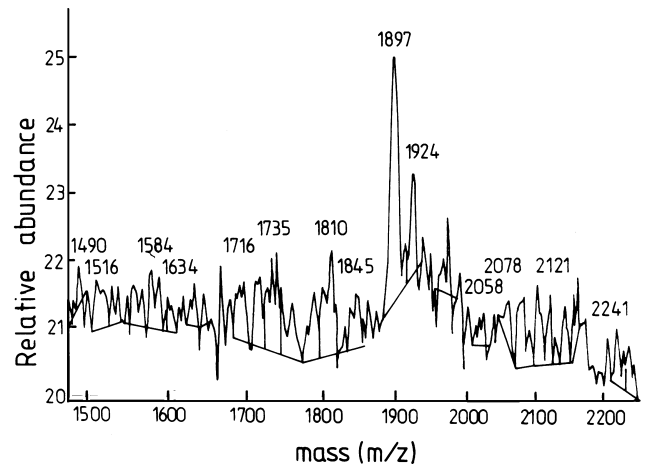


Figure 5 MALDI-MS of the major desialylated neutral oligosaccharide (structure B in Figure 4) identified in Bowes melanoma t-PA

by the exoglycosidase is based on the change in hydrodynamic volume on Bio-Gel P-4 chromatography [46]. Assignments of glycosyl residue sequence, anomeric configuration and position of glycosyl linkage are based on the specificities of the exoglycosidases and lectins used and the known structures of N-linked oligosaccharides.

The results of SLAC/GFC (summarized in Tables 1 and 2) revealed a series of complex glycans, mainly core fucosylated and bi-antennary, in which the terminal Gal residues of a typical complex N-linked glycan were replaced mainly by GalNAc. The

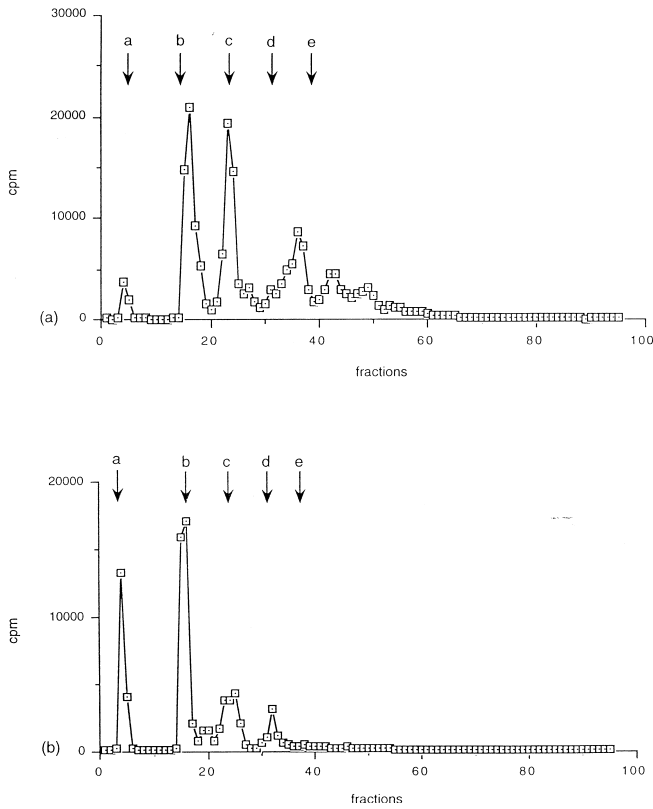


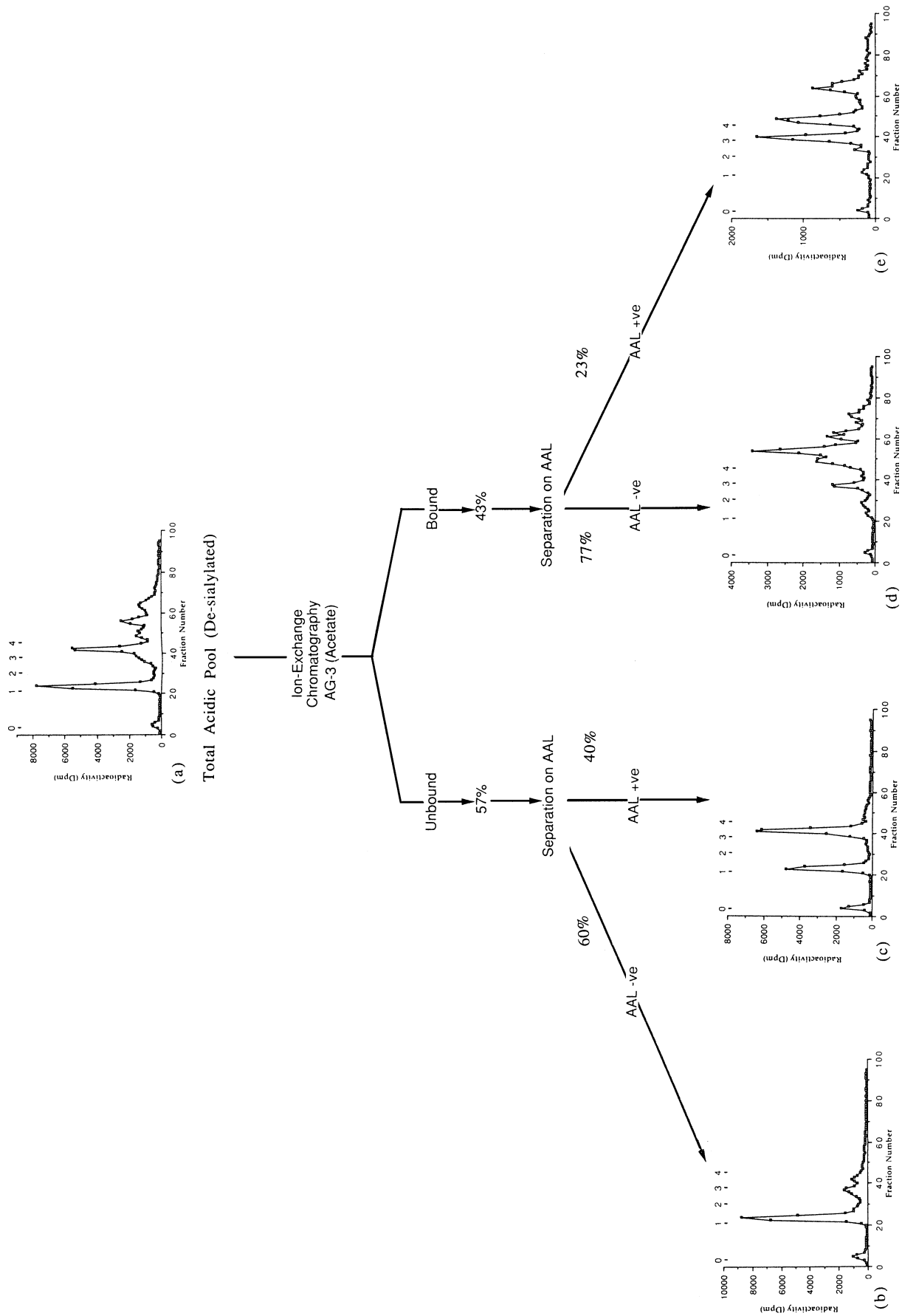
Figure 6 Anion-exchange HPLC of Bowes t-PA acidic glycans after desulphation

HPLC was performed with a Vydac 301HP575 weak-anion-exchange column as described in the Materials and methods section. Arrows a-e refer to the elution positions of standard glycans bearing 0-4 sialic acid residues respectively. (a) t-PA acidic glycans after desialylation; (b) t-PA desialylated acidic glycans after desulphation and subsequent de-esterification.

main component, eluting at 16 gu on Bio-Gel P-4 and accounting for 50 % of the neutral desialylated pool, is bi-antennary core-fucosylated with two terminal GalNAc residues (B, Figure 4). This glycan was analysed further by MALDI-MS (Figure 5). The molecular mass of 1897 Da suggests an isobaric monosaccharide composition of HexNAc₆Hex₃DeoxyHex₁ (sodium adduct), which is consistent with the proposed structure (B, Figure 4). The remaining 50 % of glycans comprised related components, some of which contained outer-arm Gal instead of or as well as GalNAc. Small amounts of tri-antennary glycans, and some structures lacking core fucose, were also detected.

Desulphation

An aliquot of the desialylated acidic glycan pool was subjected to desulphation by mild methanolysis followed by *N*-acetylation. The resulting glycans were shown to be largely neutral by both HVE and anion-exchange chromatography. De-esterification of the resulting neutral material, however, resulted in the regeneration of acidic glycans, which were separated into three charged species by anion-exchange HPLC, suggesting the presence of glycans bearing one, two and three uronic acid residues (Figure 6). More highly charged species seen before desulphation were not regenerated, suggesting that at least some of the original charge had been due to substitution with sulphate. Very little material remained neutral after de-esterification, indicating that



Scheme 2 Anion-exchange chromatograms of the total desialylated acidic glycan pool and fractions after separation on AG-3 x 4A (acetate) and AAL

Anion-exchange HPLC was performed on a Micropak AX-5 column as described in the Materials and methods section. Labels 0-4 refer to the elution positions of standard glycans bearing 0-4 sialic acid residues respectively. **(a)** Total acidic glycan pool after desialylation. **(b to e)** Acidic desialylated glycans: **(b)** non-sulphated, no core fucose; **(c)** non-sulphated, with core fucose; **(d)** sulphated, no core fucose; **(e)** sulphated, with core fucose.

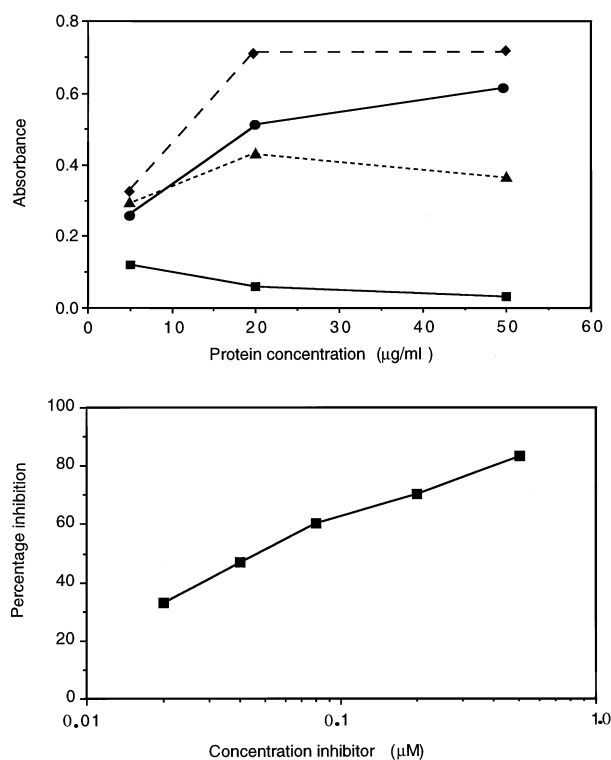


Figure 7 Detection of the L2/HNK-1 carbohydrate epitope by ELISA

Upper panel: direct binding assay. Glycoproteins were coated on to plates and tested for reaction with anti-L2/HNK-1 monoclonal antibody 336 at 1:1000 dilution. Plates were coated with: ◆, t-PA; ●, L1; ▲, tenascin-R; ■, human α 1 acid glycoprotein. Lower panel: competitive ELISA. The inhibition of binding of monoclonal antibody to plates coated with t-PA was measured after preincubation of antibody (1:1000 dilution) with different concentrations of tenascin-R as described in the Materials and methods section.

modification of glycans with sulphate alone as the sole charged species is not prevalent in t-PA. Repeated treatment with methanolic HCl failed to cause any further neutralization.

Fractionation of acidic desialylated glycans with AG3 x 4A anion-exchange resin (acetate form)

The desialylated acidic glycan pool was fractionated on an AG3 x 4A (acetate) column, under conditions in which sulphate- (or phosphate)-bearing oligosaccharides are bound whereas those carrying three carboxylic acid groups or fewer are unbound (A. J. Jaques and S. E. Zamze, unpublished work). Under these conditions, 57% of the structures were unbound (eluted with 4 M acetic acid) and 43% bound (eluted with sodium hydroxide). The unbound and bound pools from AG3 x 4A were each further separated by AAL affinity chromatography and analysed by anion-exchange HPLC (Scheme 2). Glycans carrying neither sulphate nor core fucose (not bound by either resin or AAL, 34% of the total acidic glycan pool) gave principally a single mono-charged peak, suggesting glycans bearing one uronic acid group. The non-sulphated, core-fucosylated pool (not bound by resin but bound by AAL, 23%) gave two peaks corresponding to mono- and di-(or tri-) uronic acid-containing oligosaccharides. The sulphated glycans (resin bound), either with or without core fucose (10% and 33% of the total acidic pool respectively), seem to reflect the corresponding sulphated mono-, di- and possibly tri-uronic acid-containing oligosaccharides.

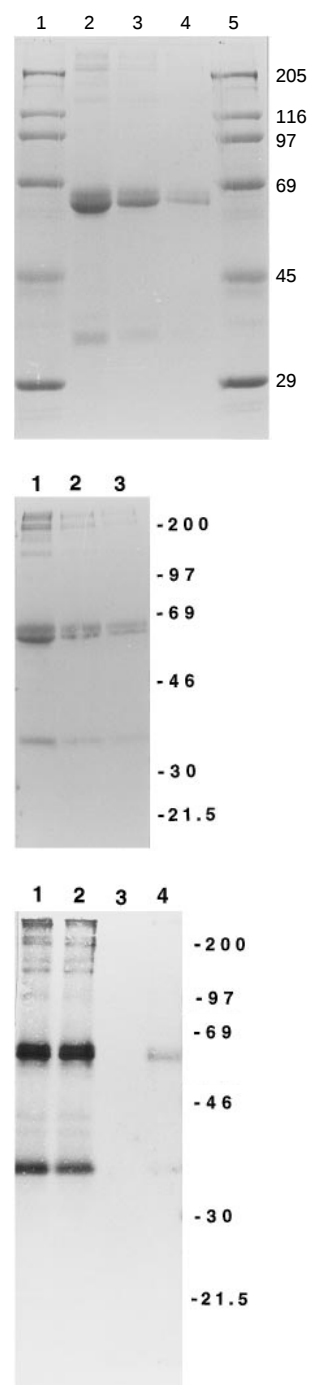


Figure 8 SDS/PAGE and Western blotting of Bowes melanoma t-PA

Proteins were separated by SDS/PAGE on 10% polyacrylamide gels and either stained with Coomassie Blue or transferred to nitrocellulose sheets followed by immunodetection with anti-L2/HNK-1 monoclonal antibodies. Top panel: Coomassie Blue-stained SDS/PAGE gel. Lanes 1 and 5 contained molecular mass markers; lanes 2–4 contained 10, 5 and 2.5 μg of t-PA respectively. Middle and bottom panels: immunoblots of Bowes t-PA. Middle panel: Lanes 1–3 contained 2.5, 1.0 and 0.5 μg of t-PA respectively. Immunodetection was performed with monoclonal antibody 336 (1:500 dilution). Bottom panel: lanes 1 and 2 contained 0.5 and 0.25 μg of t-PA respectively; lanes 3 and 4, 0.25 and 0.75 μg of t-PA after PNGase F digestion. Immunodetection was performed with monoclonal antibody 412 (1:1000 dilution). In all panels the migration positions of molecular mass rainbow markers are indicated in kDa. The lower molecular mass L2/HNK-1 reactive band migrating at approx. 35 kDa is the result of proteolytic cleavage of t-PA [16].

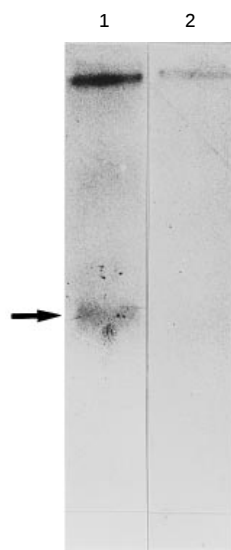


Figure 9 Autoradiograph of ^{35}S -labelled Bowes t-PA

Samples of ^{35}S -labelled immunoaffinity-purified t-PA were separated by SDS/PAGE on 15% polyacrylamide gels and detected by autoradiography. Lanes 1 and 2, t-PA before and after treatment with PNGase respectively. The arrow corresponds to the migration position of t-PA (approx. 70 kDa).

Reaction of Bowes melanoma t-PA with anti-L2/HNK-1 monoclonal antibodies

ELISA

The neural glycoproteins, L1 and tenascin-R (both known to express the L2/HNK-1 epitope), and Bowes melanoma t-PA all showed a positive reaction in ELISA with anti-L2/HNK-1 monoclonal antibodies, 336 and 412, whereas no reaction was observed with human α -1 acid glycoprotein (Figure 7, upper panel). No reaction was observed with t-PA after treatment with PNGase-F or with further control glycoproteins, transferrin, ovalbumin and horseradish peroxidase (results not shown). The binding of anti-L2/HNK-1 antibodies to t-PA could be competed out by preincubation of antibodies with tenascin-R (Figure 7, lower panel).

SDS/PAGE and Western blotting

The type I and type II variants of t-PA [13] migrated as bands of higher and lower molecular masses, respectively, of approx. 70 kDa in SDS/PAGE (Figure 8, top panel). Both of these forms reacted with anti-L2/HNK-1 monoclonal antibodies, 336 and 412 (Figure 8, middle and bottom panels). The band of lower molecular mass migrating at approx. 35 kDa is due to proteolytic cleavage of t-PA [16]. No reaction with anti-L2/HNK-1 antibodies was observed with control glycoproteins, 5 μg each of transferrin, ovalbumin, horseradish peroxidase or human α -1 acid glycoprotein, by Western blot (results not shown). As in ELISA, reaction was completely abolished by digestion of t-PA with PNGase-F (Figure 8, bottom panel), suggesting that the epitope is present on N-linked oligosaccharides.

Sulphate incorporation into Bowes t-PA

Nascent glycoproteins were isotopically labelled by incubating Bowes melanoma cells with [^{35}S]sulphate. Effective incorporation

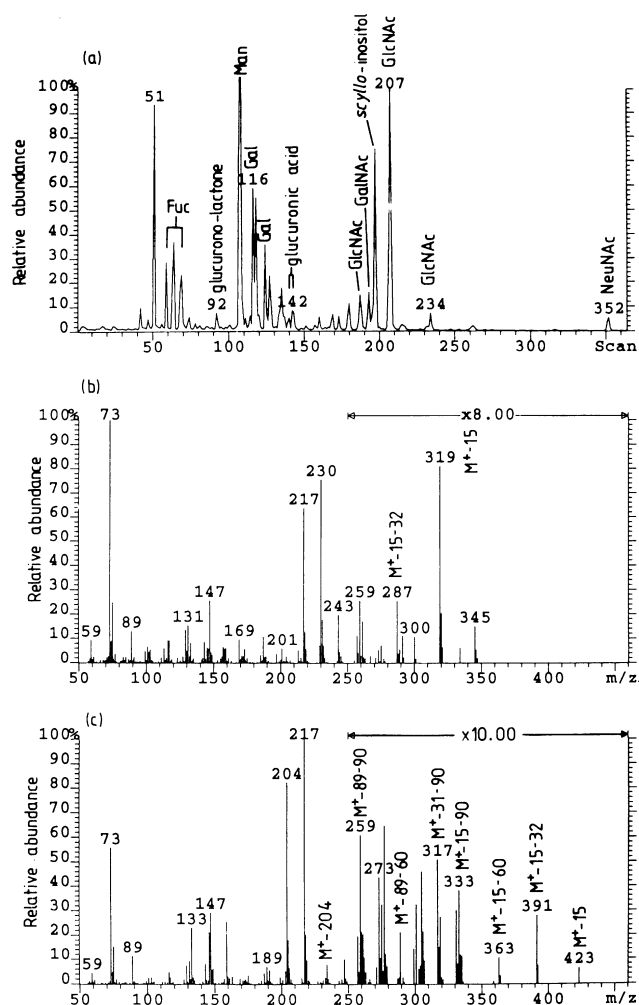


Figure 10 GC-MS monosaccharide compositional analysis of Bowes melanoma t-PA

Monosaccharides were released from t-PA (50 μg of glycoprotein) by methanolysis and analysed as their TMS 1-*O*-methylglycosides after re-*N*-acetylation. All components were identified by comparison of retention times and mass spectra with those of standard monosaccharides. (a) Total ion-current chromatogram of the trimethylsilylated methanolysis products from t-PA; (b) Electron impact (EI) mass spectrum of trimethylsilylated 1-*O*-methylglucurono-6,3-lactone from t-PA; (c) EI mass spectrum of trimethylsilylated 1-*O*-methylglucurono-6-*O*-methyl ester from t-PA.

of [^{35}S]sulphate into macromolecules could be obtained only by using sulphate-depleted medium and dialysed serum (results not shown). The effective incorporation of radiolabel specifically into t-PA was reproducibly obtained by stimulating the production of t-PA with phorbol esters [44], and was much lower in control cultures without stimulation. t-PA was purified from other labelled secreted glycoproteins by ultrafiltration and affinity adsorption as detailed in the Materials and methods section.

The purified and radiolabelled t-PA migrated at approx. 70 kDa (Figure 9, lane 1). To determine whether the isotope was incorporated into carbohydrate, t-PA was digested with PNGase F. De-*N*-glycosylation was shown to remove effectively all radioactivity from the protein (Figure 9, lane 2), suggesting that sulphate is specifically incorporated into N-linked oligosaccharides on Bowes t-PA.

Identification of glucuronic acid by GC-MS

The monosaccharide composition of the intact glycoprotein was determined by GC-MS of the TMS methyl glycosides. The constituents identified were mannose, galactose, fucose, *N*-acetylglucosamine, *N*-acetylgalactosamine, *N*-acetylneuraminic acid and glucuronic acid (Figure 10a). The retention times and mass spectra of the uronic acid TMS derivatives obtained from t-PA (Figures 10b and 10c) were identical with those obtained from authentic glucuronic acid. No other uronic acid components were detected.

DISCUSSION

The results presented show that the glycosylation of Bowes melanoma t-PA consists of neutral oligosaccharides (accounting for approx. 50% of total structures), which are principally $\text{Man}_{7-8}\text{GlcNAc}_3$, sialylated *N*-acetylgalactosamine-containing oligosaccharides (13–15%), and a further pool of acidic glycans containing glucuronic acid and/or sulphate and sialic acid (37–38%).

The analysis of the neutral desialylated complex glycans by the strategy of SLAC/GFC allows the separation of a complex mixture of oligosaccharides and the assignment of their structures. Our study confirms the results of Chan et al. [22] that the major sialylated oligosaccharide is structure B in Figure 4, with minor amounts of the non-sialylated form present. Similarly, structures C-2 and D-2 in Figure 4 are the same as those reported by Chan et al. [22]. In addition, we show the presence of a further series of related components, some containing terminal Gal instead of or as well as terminal GalNAc, some lacking core fucose, as well as a proportion of tri-antennary and hybrid components. These results suggest the presence of competing β 1,4-galactosyl and *N*-acetylgalactosaminyl transferases in the Bowes melanoma cell line. Addition of Gal was always to the $\text{Man}_{7-8}\text{GlcNAc}_3$ arm, as shown by using jack bean α -mannosidase under conditions that distinguish between $\text{Man}_{7-8}\text{GlcNAc}_3$ and $\text{Man}_{7-8}\text{GlcNAc}_6$ linkages. Our study shows no evidence for the presence of the complex structures reported by Parekh et al. [21], which would have failed to bind to WFA, RCA agglutinin, and for the most part to concanavalin A. In contrast with Chan et al. [22], we found no resistance of $\text{GalNAc}\beta$ 1-4 GlcNAc linkages to cleavage by jack bean β -*N*-acetylhexosaminidase. The use of this enzyme in exoglycosidase sequencing as described previously [21] would therefore not have distinguished between the presence of a GalNAc-GlcNAc disaccharide or two terminal GlcNAc residues.

In addition to the presence of sialylated complex glycans, and in agreement with Parekh et al. [21], our study shows the existence of acidic glycans that resist neutralization with neuraminidases. Methods of compositional analysis by GC-MS, AG-3 anion exchange, chemical desulphation by mild methanolysis followed by de-esterification, and metabolic labelling of Bowes t-PA with [^{35}S]sulphate demonstrated the presence of glycans containing glucuronic acid and sulphate. Reaction of Bowes t-PA with anti-L2/HNK-1 monoclonal antibodies is consistent with the presence of such glycans and further suggests the presence of terminal 3-sulphated glucuronic acid groups or closely related structures. Loss of L2/HNK-1 reactivity and isotopic sulphate after treatment of t-PA with PNGase F suggests that these structural groups are located on N-linked glycans. Very few of the glycans contained sulphate as their sole charged group because nearly all of the material regained a negative charge after de-esterification.

Previous analytical results [21] showed that the acidic neuraminidase-resistant glycans of t-PA were located exclusively at Asn-448. They were identified on both type I and II t-PA but

in very different amounts. In type I t-PA, where Asn-184 is occupied, they accounted for only 6% of total glycans at this site, but in type II, where Asn-184 is unoccupied, they accounted for 72%, thus providing an interesting example of site-controlled glycosylation. Western blotting performed during the present study confirmed the presence of L2/HNK-1 reactive glycans on both type I and II t-PAs. Interestingly, t-PA obtained from mouse epithelial (C127) cells also contains sulphated N-linked glycans that show a similar pattern of site distribution, being located mainly at site 448 [47].

The HNK-1 and L2 monoclonal antibodies recognize an unusual carbohydrate structure that is expressed abundantly in neural tissues and has been implicated in a number of adhesion events (see, for example, [48–50]). The presence of this epitope on Bowes t-PA is thus consistent with the expression of this molecule in a cell line of neuroectodermal origin. t-PA itself is expressed abundantly in neural tissues both during development and in the adult central nervous system. Given the prevalence of L2/HNK-1 reactive carbohydrates on neural glycoconjugates, it is highly probable that t-PA expressed in neural tissues also carries L2/HNK-1 carbohydrate. The structure of the L2/HNK-1 epitope has been characterized in detail for the neural-specific 3-sulphoglucuronylneolactosylceramides [32,51] but not for protein-bound glycans. Work is currently in progress to define the structure of the L2/HNK-1 reactive N-linked glycans on Bowes t-PA.

GalNAc-containing N-linked oligosaccharides were first identified on pituitary glycoprotein hormones [23,24]. Their synthesis on these glycoproteins requires the presence of a peptide motif Pro-Xaa-Arg/Lys (PXR/K) located six to nine residues N-terminal to the N-glycosylation site that is recognized by a PXR/K-specific GalNAc transferase [52–54]. The GalNAc-containing glycans of t-PA are located mainly at glycosylation site 448 with a small amount at site 184 [22]. The PXR/K sequence is absent from t-PA, although a very similar motif, Pro-Try-Leu-Arg, is present 10 residues towards the N-terminus from Asn-448. The presence of a separate GalNAc transferase in the Bowes melanoma cell line whose activity is independent of the PXR/K motif has been suggested [53].

Glycoproteins derived from neural tissues have not as yet been shown to express GalNAc-containing N-linked oligosaccharides, although neural tissues have been shown to express PXR/K-specific GalNAc transferase activity [53].

t-PA-catalysed plasminogen cleavage in the extracellular matrix occurs independently of the presence of fibrin, an essential cofactor in vascular fibrinolysis. Other cofactors, however, may come into play when fibrin is absent. For example, heparin, fibronectin, thrombospondin and histidine-rich glycoprotein have all been postulated to act as potential extravascular cofactors ([55] and references therein). Thrombospondin in particular seems to be associated with t-PA expression and neural development. The glycosylation of t-PA expressed in the nervous system might affect its enzymic activity, as reported for vascular fibrinolysis [56–59], or it might possibly be involved in recognition of t-PA by neuronal cell surface receptors.

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