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Tissue-specific N-glycosylation, site-specific oligosaccharide patterns and lentil lectin recognition of rat Thy-1

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To examine the extent to which protein structure and tissue-type influence glycosylation, we have determined the oligosaccharide structures at each of the three glycosylation sites (Asn-23, 74 and 98) of the cell surface glycoprotein Thy-1 isolated from rat brain and thymus. The results show that there is tissue-specificity of glycosylation and that superimposed on this is a significant degree of site-specificity. On the basis of the site distribution of oligosaccharides, we find that no Thy-1 molecules are in common between the two tissues despite the amino acid sequences being identical. We suggest, therefore, that by controlling N-glycosylation a tissue creates an unique set of glycoforms (same polypeptide but with oligosaccharides that differ either in sequence or disposition). The structures at each of the three sites were also determined for the thymocyte Thy-1 that binds to lentil lectin (Thy-1 L^+) and for that which does not (Thy-1 L^-). Segregation of intact thymus Thy-1 into two distinct sets of glycoforms by lentil lectin was found to be due to the structures at site 74. Analysis of oligosaccharide structures at the 'passenger' sites (23 and 98) suggests that either Thy-1 L^+ and Thy-1 L^- molecules are made in different cell-types or that the biosynthesis of oligosaccharides at one site is influenced by the glycosylation at other sites.

Key words: Lentil lectin/N-glycosylation/site-specificity/tissue specificity/Thy-1

Introduction

The processing pathway for N-linked oligosaccharides has been well elucidated (Kornfeld and Kornfeld, 1985). Following the transfer of a lipid-linked oligosaccharide precursor to the asparagine which is part of the tripeptide acceptor sequence Asn-Xaa-Ser/Thr, glycosidase trimming and glycosyltransferase-mediated synthesis occur (Williams and Lennarz, 1984). No complete model exists for the mechanisms which control the specificity of N-glycosylation at an individual site (Swiedler *et al.*, 1985). Characteristics of polypeptide glycosylation include (see Kobata, 1984, for examples): (i) different glycoproteins from the same cell contain different oligosaccharide structures; (ii) an individual polypeptide usually carries several different structures; (iii) many structures are found at the same glycosylation site (commonly referred to as site heterogeneity); (iv) the pattern of oligosaccharide heterogeneity at a single glycosylation site is reproducible and not random (Swiedler *et al.*, 1983, 1985); and (v) the cell-type plays a role in oligosaccharide processing (Williams and Lennarz, 1984; Sheares and Robbins, 1986). This form of post-translational modification of polypeptide therefore serves to create discrete subsets (glycoforms) of glycoproteins which have different physical and biochemical properties that may lead to functional diversity (Regoeczi *et al.*, 1979; Vaughan *et al.*, 1982; Dobre *et al.*, 1983; Parekh *et al.*, 1985; Peterson and Blackburn, 1985; Rademacher *et al.*, 1986).

Thy-1 is the smallest known member of the immunoglobulin superfamily (Williams, 1985) and in mice and rats is a major cell-surface glycoprotein of thymocytes and brain (Barclay *et al.*, 1976). In the rat the mature protein contains 111 amino acids and is attached to the membrane via a glycophospholipid tail (Low and Kincade, 1985; Tse *et al.*, 1985). The peptide sequence is identical in the brain and thymocyte forms (Williams and Gagnon, 1982; Seki *et al.*, 1985). Since the peptide is related to the immunoglobulin domain structure, which is a highly stable structure, we expect the folding and tertiary structures of the peptide to be the same in each tissue. Thy-1 is N-glycosylated at three sites (Asn-23, 74 and 98), and is therefore an ideal candidate to probe cell-type specific N-glycosylation (Figure 1). Monosaccharide analysis has shown glycosylation differences, and the exact nature of these differences (Barclay *et al.*, 1976) is established here.

Results and Discussion

Strategy of the analysis

Schemes for the purification of the various forms of Thy-1 and for the isolation of glycoproteins are illustrated in Figure 1. The purified glycoproteins were analysed by SDS–PAGE (Barclay *et al.*, 1976). Glycopeptides were prepared by trypsin digestion of intact Thy-1 and isolated by gel-permeation chromatography and reverse-phase h.p.l.c. as described in the legend to Table I. The location on the polypeptide sequence of each glycopeptide was determined by comparison of its amino acid composition with the known primary amino acid sequence of Thy-1. Oligosaccharides were released from the whole glycoprotein and the individual glycopeptides by hydrazinolysis and the reducing termini of the released structures were radioactively labelled by reduction with NaB^3H_4. One aliquot of each labelled oligosaccharide mixture was subjected to high-voltage paper electrophoresis to fractionate structures on the basis of charge density, while a second was subjected to exhaustive neuraminidase digestion followed by gel-filtration on a Bio-Gel P-4 (−400 mesh) column which separates structures on the basis of hydrodynamic volume. The structures of carbohydrates within each peak were then determined by a combination of sequential exoglycosidase digestion and methylation analysis (Materials and methods) and as described elsewhere (Parekh *et al.*, 1985; Taniguchi *et al.*, 1985; Ashford *et al.*, 1987). Representative data of the detailed structural analysis are found throughout the text. At the end of the analysis the sum of the structures from the glycopeptides was compared to structures from the whole Thy-1 preparations.

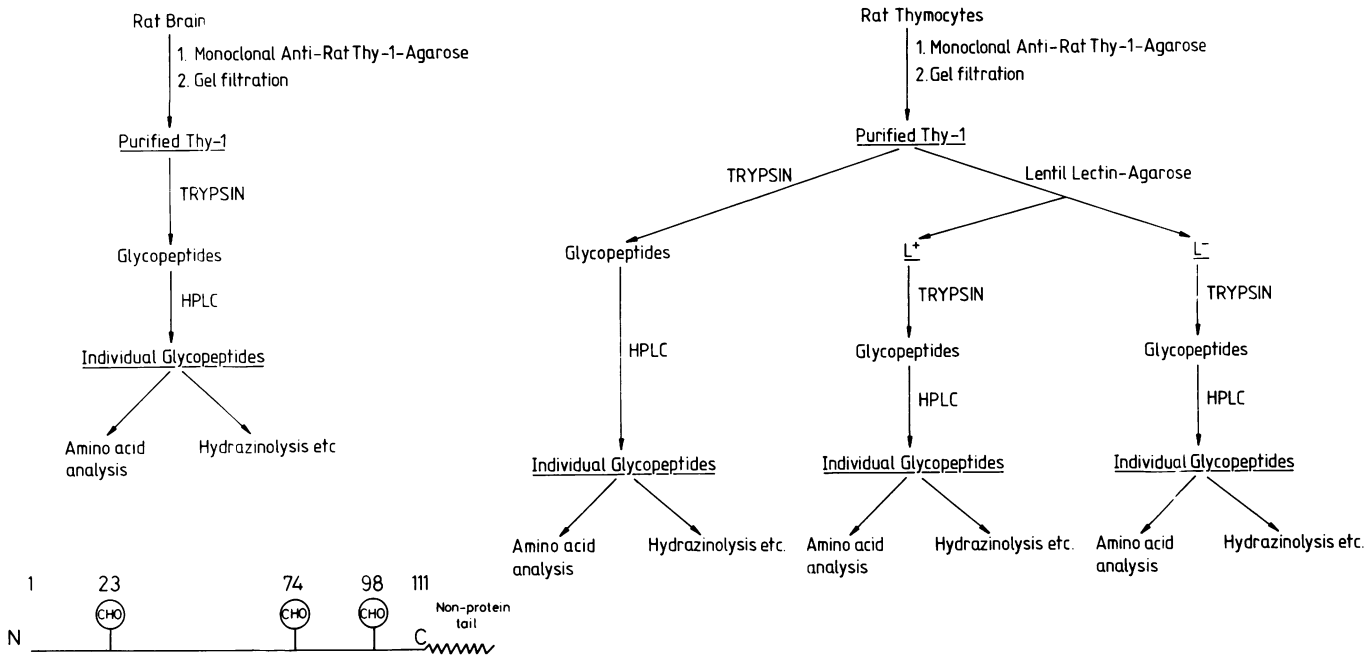

Fig. 1. Strategy for purification and fractionation of Thy-1 and glycopeptides from rat brain and thymocytes.

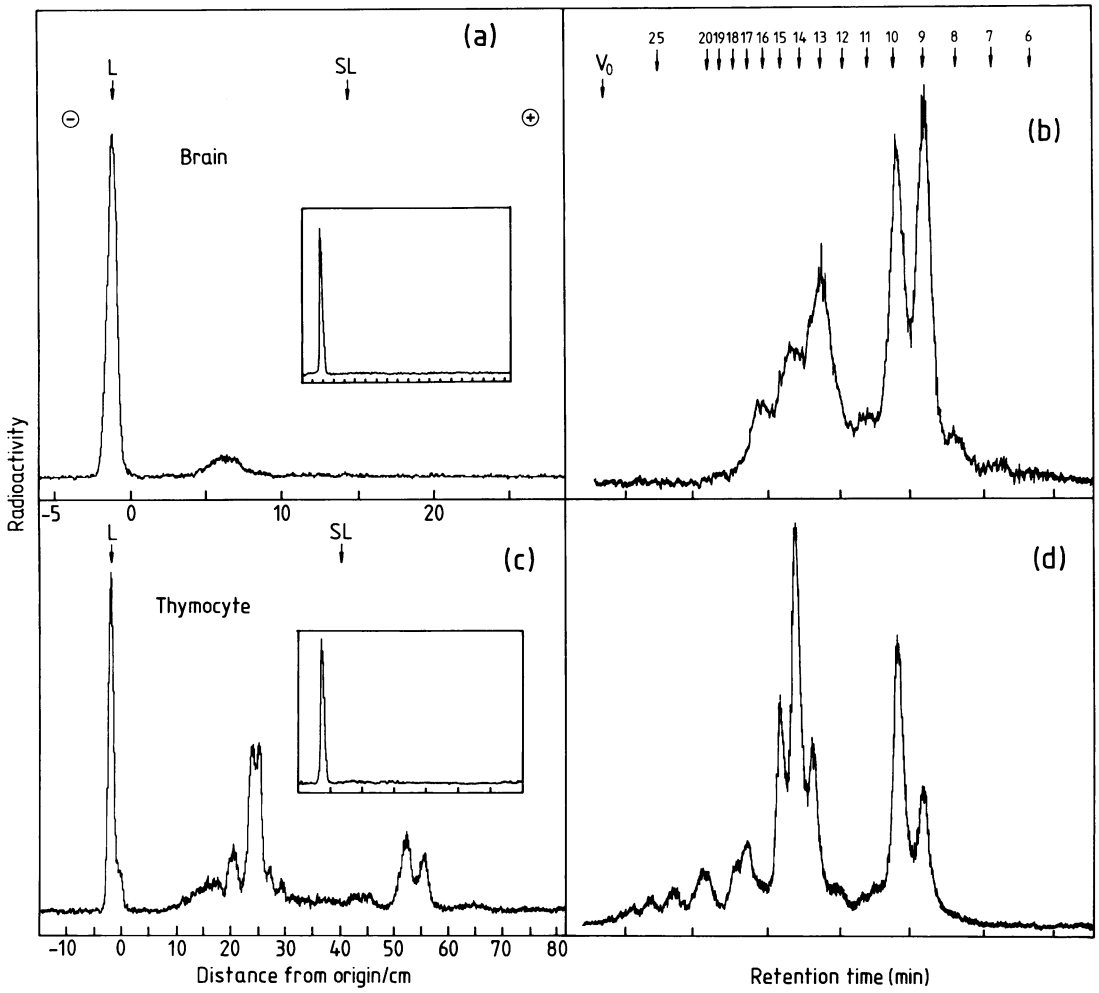

Fig. 2. Representative high-voltage electrophoretograms (**a**, **c**) and Bio-Gel P-4 (−400 mesh) gel permeation chromatograms (**b**, **d**) of the oligosaccharides attached to brain (**a**, **b**) and thymocyte (**c**, **d**) Thy-1. Arrows indicate the positions to which lactitol (L) and 3(6)′ sialyllactitol (SL) migrated. The percentage of each was: neutral, brain, 88%; thymocyte, 38%; acidic, brain, 12%; thymocyte, 62%. Inserts a, c are the high-voltage electrophoretograms of the ^{3}H-labelled oligosaccharides after exhaustive neuraminidase digestion.

Table I. Amino acid compositions of glycopeptide pools

Glycopeptide	h.p.l.c peaks	D	T	S	E	P	G	A	V	M	I	L	Y	F	H	K	R	Asn[a] site	% of site	Recovery of site (%)
Brain																				
aB I	3	3.3	1.1	0.5	2.8	0.9	0.8	0.6	0.3	0.1	0.9	1.4	0.1	1	1.6	0.3	0.3	23	31	54
B I	1	3.2	1.2	0.5	3.4	1.1	0.6				1.4	1.3		1	1.9			23	69	
C II	1	1.2	2.3		0.4	0.1	0.2	0.9	0.9			1.8		1.0		1		74	26	61
C III	1	1.0	2.6					1.0	1.1			1.8		1.0		1		74	74	
C I	1	2.2	1.0	2.7	1.2	1.0	1.2		1.6		0.3	0.3				1		98	39	56
D I	1	2.2	1.0	2.9	1.3	1.0	1.3		1.5							1		98	61	
Thymus																				
B II	4	3.4	1.2	0.7	3.1	0.9	0.8	0.5	0.3		0.9	1.1		1	1.6	0.3		23	38	45
C II	1	3.2	1.0	0.4	2.7	0.6	0.3				1.1	1.1		1	2.0			23	62	
B III	2	1.3	2.7	0.2	0.4		0.4	1.0	0.9			1.8		0.9	0.2	1		74	55	62
C III	1	1.2	2.7	0.2	0.2	0.1	0.2	0.9	1.4		0.1	1.7		0.9	0.3	1		74	45	
B I	1	1.9	0.8	2.4	0.9	0.7	1.0		1.0							1		98	33	64
C I	1	2.0	0.8	2.3	1.1	0.9	1.0		1.1			0.2	0.2			1	0.2	98	67	
Thymus (L^-)																				
B II	2	3.1	1.3	0.5	3.0	0.9	0.6	0.2	0.4		0.9	1.4		1	1.6	0.2		23	25	50
C II	1	3.4	1.1	0.2	3.1	1.0	0.2				0.9	1.0		1	1.9			23	75	
B III	2	1.1	3.0	0.2	0.2		0.3	1.0	1.0			2.0		1.0		1		74	80	66
C III	2	2.4	2.8	1.1	1.2	0.5	0.9	1.1	0.8		0.6	2.3	0.2	1.1	0.6	1	0.4	74	20	
B I	2	2.0	0.9	2.3	1.1	0.6	1.4		0.9						0.1	1	0.1	98	29	64
C I	2	2.5	1.0	2.7	1.4	0.9	1.1		0.8	0.1		0.2	0.2			1	0.2	98	71	
Thymus (L^+)																				
B II	5	3.3	1.1	0.9	3.2	0.7	1.4	0.4	0.3		0.9	1.1		1	1.2	0.3		23	69	55
C II	1	3.2	1.0	0.2	3.1	1.2	0.3	0.4			1.0	1.3		1	1.7			23	31	
B III	1	1.2	3.3	0.3	0.3		0.4	1.3	1.1			2.0		1.0		1		74	59	58
C III	1	1.1	2.8	0.2	0.3		0.2	1.0	0.8			1.7		0.9		1		74	41	
B I	1	2.4	1.0	3.1	1.3	1.1	1.6		0.9							1		98	38	61
C I	1	2.3	1.0	2.8	1.2	[b]	1.2		1.0			0.1				1		98	62	
Expected composition		D	T	S	E	P	G	A	V	M	I	L	Y	F	H	K	R			
		3	1		3	1					1	1		1	2			23		
		1	3					1	1			2		1		1		74		
		2	1	3	1	1	1		1							1		98		

ZRVISLTACLVNQNLRLDCRHENNTNLPIQHEFSLTREKKKHVLSGTLGVPEHTYRSRVNLFSDRFIKVLTLANFTTKDEGDYMCELRVSGQNPTSSNKTINVIRDKLVKC

[a]Peaks judged to be from the same site in each h.p.l.c. run were pooled as shown in the table under 'h.p.l.c. peaks'. These peaks containing the same glycopeptide were eluted from the h.p.l.c. in clusters and the Roman numerals indicate the order of elution of each cluster. The positions of cleavage for the different sites is shown on the Thy-1 sequence and it should noted that the site 23 glycopeptide was derived from a chymotryptic-like cleavage after Phe-33. Thus compositions for site 23 pools were normalized to Phe = 1 while Lys = 1 was used for the other sites. Contamination of one site with another can be assessed by comparing compositions with the expected compositions shown above and on this basis the only clear-cut contamination was in CIII of Thymus L^- (site 74) which is likely to be 25% contaminated with site 23 glycopeptides. Smaller amounts of contamination may also be present in peptides aB I (Brain), B II and C III (Thymus), B II (Thymus L^-) and B II (Thymus L^+) but an assignment to one contaminating peptide could not be clearly made. The recovery of glycopeptides is given as percentage of starting material. Total recovery of a site (%) was obtained by addition of glycopeptides containing the same N-substituted Asn residue.

[b]Pro was present but not quantitated due to baseline drift on the 440-nm trace.

N-glycosylation patterns of whole Thy-1 are tissue-specific

Electrophoretograms and Bio-Gel P-4 profiles for carbohydrates from brain and thymus Thy-1 are shown in Figure 2 and lead to the following conclusions. (i) Almost all of the brain Thy-1 oligosaccharides carry no charge while the majority of thymus Thy-1 oligosaccharides are negatively charged. (ii) The negative charge on the Thy-1 oligosaccharides is due only to sialic acids, since all oligosaccharides convert to neutral species after digestion with neuraminidase (Insert Figure 2a, c). (iii) High-voltage electrophoresis reveals a large number of discrete structures from thymus Thy-1 but these carry only one or two sialic acids, as judged by anion-exchange chromatography (Insert Figure 3a, c).

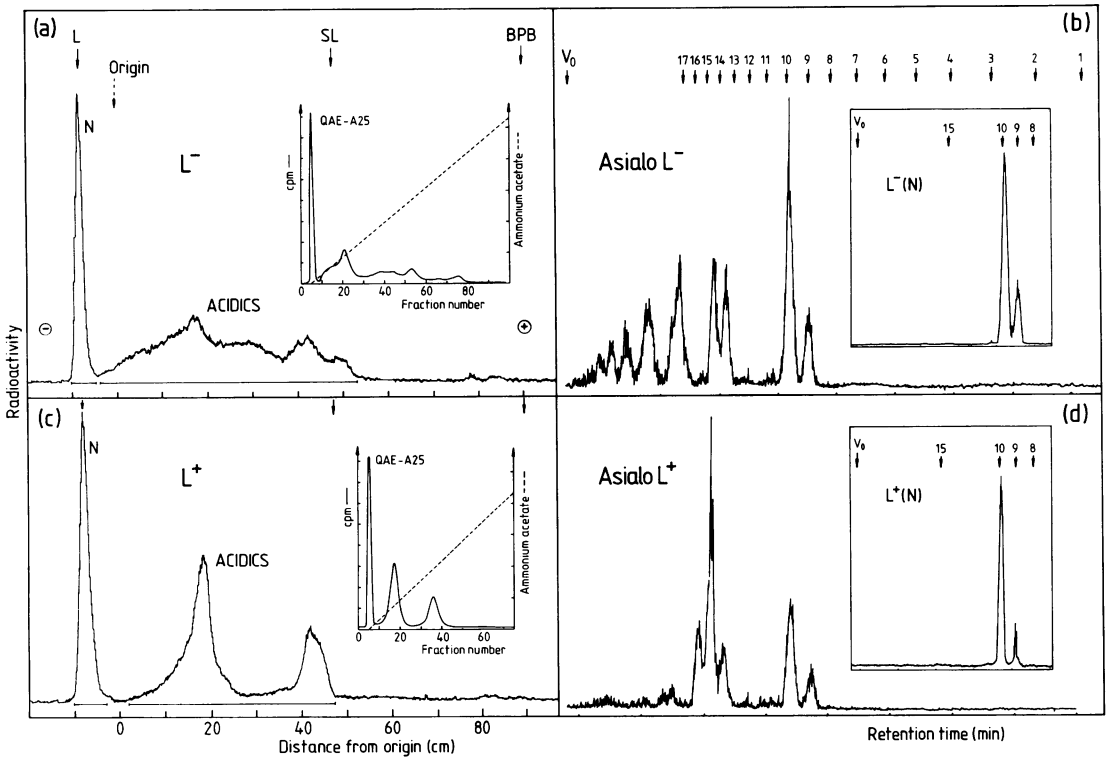

Fig. 3. (**a**, **c**) Paper electrophoretograms of the radioactive oligosaccharides released from lentil lectin unbound (L^-) and lentil lectin bound (L^+) thymus Thy-1. The radioactive oligosaccharide mixtures were subjected to high-voltage paper electrophoresis as described in Materials and methods. Arrows indicate the positions to which lactitol (L), 3(6)′ sialyllactitol (SL) and bromophenol blue (BPB) moved. The incidence of structures found were: (**a**) L^-, neutral, 26%; acidic, 74%; (**c**) L^+, neutral, 29%; acidic, 71%. The samples were also applied to a QAE-A25 Sephadex column (6 mm × 10 cm) in 2 mM ammonium acetate (pH 5.3) and the column developed with a 2–350 mM linear gradient of ammonium acetate pH 5.4 (inserts a, c). The acidic oligosaccharides in L^+ eluted in peaks corresponding to the standard elution positions of monosialylated and disialylated oligosaccharide. The acidic oligosaccharides from L^- did not elute as resolved species suggesting interaction with the column matrix. (**b**, **d**) Bio-Gel P-4 column chromatograms of the asialo oligosaccharides liberated from thymocyte Thy-1 L^+ and Thy-1 L^- and of the naturally occurring neutral oligosaccharides (N) (insert, b, d).

Since the separation of oligosaccharides by high-voltage electrophoresis depends both on their charge and on their mass, most individual peaks must therefore correspond to oligosaccharides differing with respect to size and not charge. This is consistent with the complexity of the Bio-Gel P-4 profile of the asialo oligosaccharide mixture from thymocyte Thy-1 (Figure 2d). (iv) The set of asialo oligosaccharides generated from brain Thy-1 differs from that generated from thymus Thy-1 as revealed by the Bio-Gel P-4 gel-filtration chromatography (Figure 2b).

When the structures of the peaks from the Bio-Gel P-4 separations were determined (Table II) the following conclusions were drawn. (i) Thy-1 molecules from both tissues contain oligomannose structures (see Figure 4b, f). (ii) All structures other than oligomannose ones differed between brain and thymus Thy-1. (iii) All complex structures from thymus Thy-1 (biantennary, triantennary, tetraantennary and lactosaminoglycans) contain at least one terminal sialic acid (Figure 2b, d) and the majority of the other non-reducing termini are α-galactose (Table III). The fact that all complex structures are sialylated is shown by the finding that only oligomannose structures are seen on the Bio-Gel P-4 profiles of the neutral oligosaccharides from thymus Thy-1 L^+ and thymus Thy-1 L^- (see inserts Figure 3b, d). (iv) Brain non-oligomannose structures include non-bisected hybrid-type and incompletely defined structures, at least some of which are bisected and terminate with outer-arm fucose residues (i.e. Galα(β)1→3(4)[Fucα1→3(4)]GlcNAc→R) (see Figures 4 and 5 and Table III). These latter structures were resistant to exoglycosidase digestion (see Materials and methods) with the exception of α-fucosidase which removed the core α1-6 linked fucose residue (Figure 4g, h). Figure 5 shows a ^1H n.m.r. spectrum of the asialo oligosaccharides from brain Thy-1 eluting at 12.8 g.u. (fraction F in Figure 4g) indicating the heterogeneous environment of the fucose.

Comparison of site-specific glycosylation patterns of brain and thymus Thy-1

Analysis of glycopeptides from brain and thymus Thy-1 showed that all sites contained multiple oligosaccharide structures with clear-cut differences between the three sites in each form of Thy-1 (Table II, Figures 6 and 7). The conclusions are: (i) site 23 of thymus Thy-1 contains mainly oligomannose structures (Man-5,6), with smaller amounts of complex types (some of which are core-fucosylated) and non-fucosylated polylactosaminoglycans. In brain Thy-1 site 23 contains only oligomannose structures (Man-5,6); (ii) site 74 of thymus Thy-1 contains complex and polylactosaminoglycan types both of which are completely core-fucosylated (see Figure 4c, d for the fucose analysis of oligosaccharide structures on L^- BIII), whereas site 74 of brain Thy-1 contains completely core-fucosylated oligosaccharides (Figure 4g, h); (iii) site 98 of thymus Thy-1 contains non-fucosylated complex and lactosaminoglycan types, whereas brain Thy-1 contains either hybrid or oligomannose (Man-5)-type oligosaccharides at this site.

These data support a theory of site-specific glycosylation and not one of molecular uniformity (Bayard *et al.*, 1982). In addition it is not the case that sites which are more N-terminal are more highly processed (Pollack and Atkinson, 1983) since site 23 in both brain and thymus Thy-1 contains the least processed

Table II. Distribution of oligosaccharides on intact Thy-1 and on glycopeptides

Glycoprotein	Site	Percent of site	Complex (N)	Complex (A)	Lactos-aminoglycan (N)	Lactos-aminoglycan (A)	Oligo-mannose	Hybrid	Neutral (total) (N)	Acidic (total) (A)	Fucose (core)	αGal	Antennae
B Thy-1	–	–	23[a]	12[a]	–	–	49	16[a]	88	12			
T Thy-1	–	–	51 (N+A)	–	20 (N+A)	–	29	–	38	62			
T L^-	–	–	21 (N+A)	–	51 (N+A)	–	27	–	26	74			
T L^+	–	–	73 (N+A)	–	–	–	27	–	29	71			
Glycopeptide													
B aBI	23	31	–	–	–	–	100	–	100	–	–	–	Man6 > Man5
BI	23	69	–	–	–	–	100	–	100	–	–	–	Man6 > Man5
CII	74	26	56[a]	44[a]	–	–	–	–	56	44	+	–	Bisect
CIII	74	74	100[a]	–	–	–	–	–	100	–	+	–	Bisect
CI	98	39	–	–	–	–	5	95[a]	100	–	–	–	–
DI	98	61	–	–	–	–	78	22[a]	100	–	–	–	Man5
T BtII	23	38	18	30	3	8	42	–	62	38			
CtII	23	62	–	–	–	–	100	–	100	–			
BtIII	74	55	11	40	23	26	–	–	34	66			
CtIII	74	45	41	30	6	4	19	–	66	34			
BtI	98	33	5	77	10	8	–	–	15	85			
CtI	98	67	21	72	–	7	–	–	22	78			
L^- BII	23	25	50 (N+A)	–	50 (N+A)	–	–	–	25	75	–	±	bi
CII	23	75	–	–	–	–	100	–	100	–	–	–	Man6 > Man5
BIII	74	80	–	–	10	90	–	–	10	90	+	±	tri
CIII[b]	74	20	21	54	–	–	25	–	46	54	+	±	tetra, tri
BI	98	29	24 (N+A)	–	76 (N+A)	–	–	–	14	86	–	±	tri, bi
CI	98	71	33	67	–	–	–	–	33	67	–	±	tetra, tri, bi
L^+ BII	23	69	10	34	–	–	56	–	66	34	+	–	bi, Man6 > Man5
CII	23	31	–	–	–	–	100	–	100	–	–	–	Man6 > Man5
BIII	74	59	15	85	–	–	–	–	15	85	+	–	bi, tri (trace)
CIII	74	41	30	70	–	–	–	–	30	70	+	+	bi, tri (trace)
BI	98	38	24	76	–	–	–	–	24	76	–	–	bi, tri (trace)
CI	98	62	20	80	–	–	–	–	20	80	–	+	bi, tri (trace)

[a]Some of these oligosaccharide structures terminate with fucosylated outer-arm structures as determined by n.m.r. spectroscopy and methylation analysis and are presently under investigation (i.e. Gal$\alpha(\beta)$1→3(4)[Fucα1→3(4)]GlcNAc→R) (see Figures 4,5 and Table III).
[b]This peptide pool is like to contain ~25% contamination with site 23 glycopeptide.

structures. It is also interesting to note that the oligosaccharide site adjacent to the lipid bilayer (site 98) contains charged oligosaccharides in thymus Thy-1 and neutral ones (hybrid, oligomannose) in brain Thy-1. Finally, the charged structures on brain Thy-1 occur only at site 74 (see Figure 7 and Table III).

Separation of glycoforms by lentil lectin chromatography

Chromatography on a *Lens culinaris* (lentil) lectin–agarose column separates thymus Thy-1 into two fractions called Thy-1 L^+ and Thy-1 L^- that do not overlap in analysis on SDS–PAGE (Tse *et al.*, 1985). On the basis of glycopeptide studies (Kornfeld *et al.*, 1981) it is known that lentil lectin has high affinity for structures with fucose linked at the C-6 position of GlcNAc-1 (i.e. core-fucosylation). Fucosylated triantennary structures containing outer chains linked at C-2 and C-4 of Manα1 → 3(6) residue do not interact. The structures at each of the three sites of Thy-1 L^+ and Thy-1 L^- were analysed so as to determine the basis of this separation and to compare an intact glycoprotein fractionation with the glycopeptide data.

High-voltage radioelectrophoretograms and Bio-Gel P-4 profiles of the asialo oligosaccharides of Thy-1 L^+ and Thy-1 L^- are shown in Figure 3a–d and it is apparent that there are major differences between the two forms. The complete site analysis is summarized in Table II and Figure 7. Core-fucosylation is found on all oligosaccharides at site 74 of Thy-1 L^+ and Thy-1 L^- (see Figure 4c, d) and on all complex structures at site 23 of Thy-1 L^+ but not Thy-1 L^-. Site 98 was devoid of core-fucosylated structures in both cases. Thus every thymus Thy-1 molecule satisfies the requirement of core-fucosylation on at least one site (74) and some at two (23, 74).

The Thy-1 L^+ site 74 structures are biantennary (major) or triantennary (minor) with 2,6-linked outer chains at the Man α1-6 residue. All of these structures would be expected to bind to lentil lectin (Kornfeld *et al.*, 1981). Therefore, the interaction of Thy-1 L^+ with lentil lectin is only via the structures at site 74 in most (70%) molecules and may also involve structures at site 23 in the remaining L^+ forms (30%).

Differences other than core fucosylation at site 74 must account for the Thy-1 L^- population. At site 74 in Thy-1 L^- all structures are multiantennary with most being triantennary. Sequential exoglycosidase digestion (data not shown) and methylation analysis showed that these triantennary structures contain 2,4-linked outer chains located on the α1–3 mannose residue. This linkage would be expected to prevent lentil binding. The amount of tetraantennary oligosaccharides present at site 74 of Thy-1 L^- was too small for complete structural analysis. Obviously the structure of the outer chains must be such that no interaction with lentil lectin occurs. Further, the interaction of tetraantennary glycopeptides is complicated and additionally dependant on the presence and positioning of sialic acid (Samor *et al.*, 1986).

To conclude, the Thy-1 L^+ and Thy-1 L^- forms are segregated into two populations by lentil lectin chromatography on the basis of the position of attachment of the outer chains to the structures at site 74.

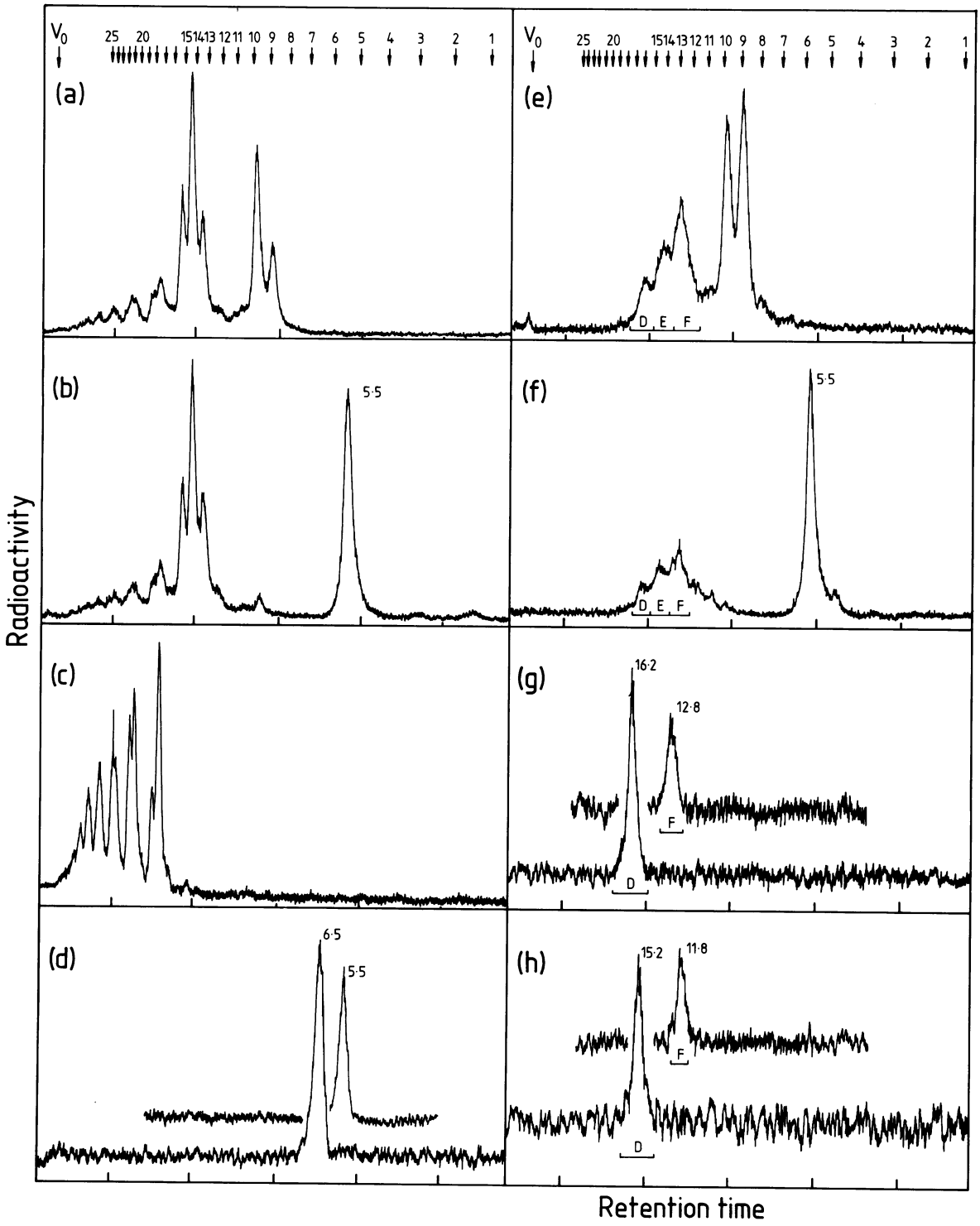

Fig. 4. Bio-gel P-4 column chromatograms of the asialo oligosaccharide fractions. The arrows at the top indicate the elution positions of glucose oligomers. Asialo oligosaccharides from thymocyte Thy-1 before (**a**) and after (**b**) jack bean α-mannosidase digestion. Asialo oligosaccharides derived from glycopeptide Thy-1 L^- BIII (site 74) before (**c**) and after (**d**) digestion (6.5 gu) with an exoglycosidase mix containing coffee bean α-galactosidase, jack bean β-galactosidase, jack bean β-*N*-acetylhexosaminidase and jack bean α-mannosidase. Digestion of the 6.5 gu fraction with bovine epididymis α-fucosidase resulted in a fraction eluting at 5.5 gu indicating the loss of one fucose residue [insert (d)]. Asialo oligosaccharides from brain Thy-1 before (**e**) and after (**f**) jack bean α-mannosidase. Fractions D, E (data not shown) and F were resistant to all exoglycosidases (see Materials and methods) except α-fucosidase which removed one core α1-6 linked fucose residue (**h**).

Terminal non-reducing α-linked galactose residues and polylactosaminoglycans

The occurrence of α-linked galactose residues (Geyer *et al.*, 1984; Spiro and Bhoyroo, 1984; Van Halbeek *et al.*, 1985) and polylactosaminoglycans (Margolis *et al.*, 1986) has been reported in a number of glycoproteins and glycolipids (Feizi, 1985). The α-linked galactose residues were first observed in glycoconjugates with blood group B activity. Both Thy-1 L^- and Thy-1 L^+ contained oligosaccharides which terminate with α-linked galactose residues (Galα1 → 3Gal → R, determined by digestion with cof-

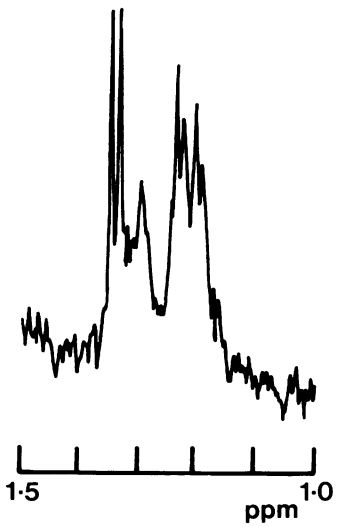

Fig. 5. 500 MHz ^{1}H n.m.r. spectrum of the asialo oligosaccharides from brain Thy-1 eluting at 12.8 gu (fraction F in Figure 4g). Fucose methyl protons resonate in the region 1.2−1.4 p.p.m.

fee bean α-galactosidase and methylation analysis, (see Table III) which are located exclusively on the Manα1-6 side (determined by sequential exoglycosidase digestion, data not shown). Such arm-specificity has also been reported for mouse thymus Thy-1. The polylactosaminoglycans are restricted to Thy-1 L^- where they are linked exclusively to the Manα1-3 side. The occurrence of both polylactosaminoglycans and non-reducing, terminal, α-linked galactose residues as found here has only been reported to occur on the four N-glycosidically linked oligosaccharides in the glycoproteins of Friend murine leukemia virus produced by Eveline cells (Geyer *et al.*, 1984).

The lack of polylactosaminoglycans in brain Thy-1 is consistent with the finding that these structures do not occur in postnatal rat brain (Margolis *et al.*, 1986). Their presence in rat, however, has been reported in neurons of sympathetic origin outside of the central nervous system (Margolis *et al.*, 1986). The presence of (3,4 di-O)-substituted *N*-acetylglucosamine, (3,6-di-O)-substituted galactose and a high fucose content are major features of the N-glycopeptides of rat brain (Krusius and Finne, 1977, 1978). Since Thy-1 is a major cell-surface glycoprotein in this tissue it is not unexpected that the non-oligomannose oligosaccharides terminate with fucosylated outer-arm structures (see Table III and Figures 4,5).

Reconstruction of Thy-1 from glycopeptides

To determine the relative frequency of individual glycoforms it is necessary to determine the precise molar amount of each oligosaccharide at a given site. Proteolytic or chemical fragmentation of glycoproteins often results in partial cleavage to give a number of peptides containing the same glycosylation site. In addition, glycopeptides of identical amino acid sequence may fractionate if several oligosaccharide sequences are attached. Table I lists the recoveries of the various peptide pools which contain single glycosylation sites. These site 'pools' were constructed from multiple peaks following reverse-phase h.p.l.c. analysis. Examination of the glycopeptide amino acid and oligosaccharide sequence data presented in Tables I and II provides an explanation for their separation by either size or h.p.l.c. absorbance. Figure 6 compares the glycosylation of the intact proteins with that of the 'reconstructed' protein. The close agreement indicates that our site analysis is essentially correct. The

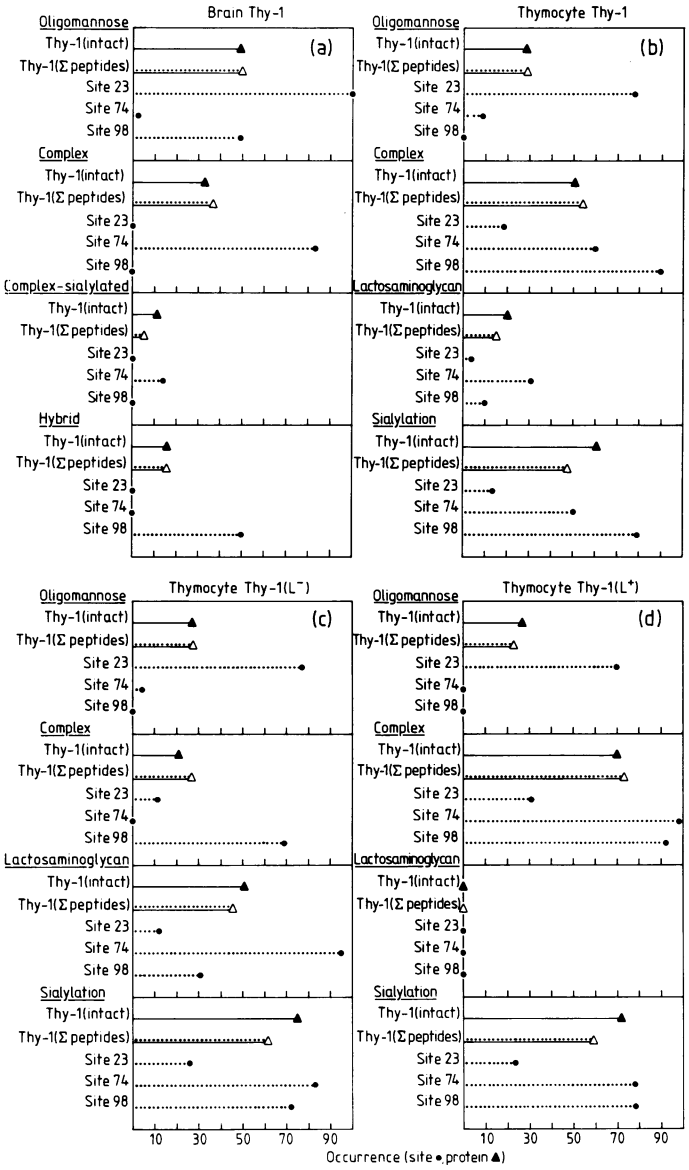

Fig. 6. Site distribution of various oligosaccharide parameters present on the glycoproteins and glycopeptides. The occurrence of a particular form of oligosaccharide at specific sites (..... ●) was obtained by a weighted summation of the sequence information from individual glycopeptides (see Table II). The peptides..... (Δ) allows a comparison of the glycosylation of the 'reconstructed' protein (i.e. the sum of the individual glycosylation sites) with that from the analysis on the intact protein (____▲). (**a**) brain Thy-1, (**b**) thymocyte Thy-1, (**c**) thymocyte Thy-1 (L^-), (**d**) thymocyte Thy-1 (L^+).

site distribution data in Figure 6 and Table II allows us to compile a set of composite glycoforms based on the oligosaccharide characteristics in Figure 7. Any actual glycoform would be derived from a particular one of these composites. The occurrence (probability) of these composite forms is shown in Figure 7 and is calculated by multiplying the frequency of each type of different structural set at each of the three sites.

Conclusion

Species-specific differences in the structures of oligosaccharides of glycoproteins have been well documented (Kobata, 1984). Similarly, glycoproteins secreted eutopically and ectopically by tumour cells *in vivo* (Yamashita *et al.*, 1981a; Mizuochi *et al.*,

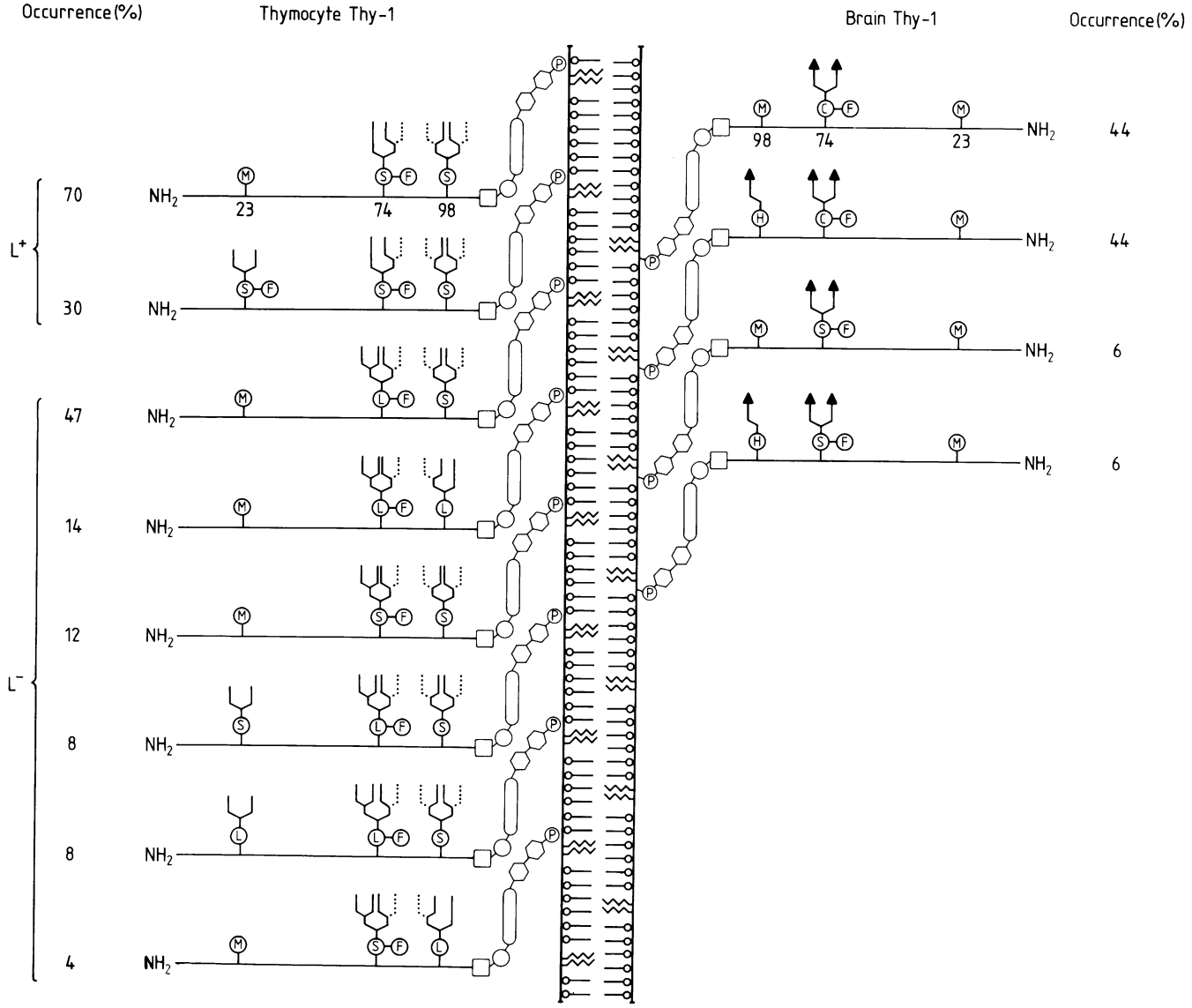

Fig. 7. 'Composite' glycoforms found in brain and thymus Thy-1. The occurrence of each composite glycoform was calculated from the site distribution data assuming that the glycosylation at each site was independent of the other sites. The Manα1 → 3Manβ → R branch is drawn to the left and the Manα1 → 6 Manβ → R branch on the right, (i.e. $_3$ ⟨ $_6$). Symbols represent the type of structures present at each site: Ⓜ, oligomannose; Ⓒ, bisected complex; Ⓢ, sialylated; Ⓕ, α-fucosyl residue linked at C-6 of the reducing terminal *N*-acetylglucosamine; Ⓛ, polylactosaminoglycan; Ⓗ, hybrid; ⟨ biantennary; ⟨ , biantennary with a trace of triantennary with 2,4-linked outer-arm branching, (i.e. $^2_{3\ 6}$⟨^{4}); ⟨ biantennary with a trace of triantennary with 2,6-linked outer arm branching; ⟨ biantennary with trace of triantennary with either 2,4 or 2,6-linked outer arm branching; ⟨ triantennary with 2,4-linked outer arm branching and trace of tetraantennary with 2,6-linked outer arm branching; ↑ denotes presence of 3,4-disubstituted outer-arm *N*-acetylglycosamine; ⬡—Ⓟ, phosphatidylinositol; □, C-terminal amino acid; ○, ethanolamine; ⬭⬡, glycans (Low *et al.*, 1986).

1983) and by cell lines *in vitro* (Yamashita *et al.*, 1984; Pierce and Arango, 1986) often show glycosylation characteristics which are cell type-specific (Sheares and Robbins, 1986). These changes however may reflect either a mutational event or a genetic polymorphism (Nishimura *et al.*, 1986). Organ-specific differences in the sugar chains of cell-surface glycoproteins produced *in vivo* have also been reported although it has not been established whether these changes are secondary to the expression of different isozymes (Yamashita *et al.*, 1985), or due to differences in the processing and subsequent folding of the precursor polypeptide (Yamashita *et al.*, 1983).

Our analysis of the *in vivo* glycosylation patterns of the same peptide isolated from two tissues confirms the tissue-specificity of the biosynthetic process. This implies that although the peptide exerts an influence on its glycosylation, the expression or exposure to glycosidases and glycosyltransferases mediate the tissue-specific characteristics (glycotype). In the case of Thy-1 we would expect the folding and tertiary structures of the peptide to be the same in each tissue since the peptide is related to the immunoglobulin domain which is a highly stable structure. However, it should be noted that Thy-1 contains a glycophospholipid membrane anchor which shows some com-

Table III. Methylation analysis of the oligosaccharides released from thymocyte and brain Thy-1.

Methylated sugar	Molar ratio[a]	
	Thymocyte	Brain
Fucitol[b]		
2,3,4-Tri-*O*-methyl (1,5-di-*O*-acetyl)	0.6	0.4
Galactitol		
2,3,4,6-Tetra-*O*-methyl (1,5,-di-*O*-acetyl)	0.7	0.4
2,4,6-Tri-*O*-methyl (1,3,5-tri-*O*-acetyl)	0.9	n.d.
2,3,4-Tri-*O*-methyl (1,5,6-tri-*O*-acetyl	0.7	n.d
Mannitol		
2,3,4,6-Tetra-*O*-methyl (1,5-di-*O*-acetyl)	0.9	1.5
3,4,6-Tri-*O*-methyl (1,2,5-tri-*O*-acetyl)	1.5	1.1
2,4,6-Tri-*O*-methyl) (1,3,5-tri-*O*-acetyl)	n.d.	0.1
2,3,4-Tri-*O*-methyl 1,5,6-tri-*O*-acetyl	n.d.	trace
3,6-Di-*O*-methyl (1,2,4,5-tetra-*O*-acetyl)	0.1	trace
3,4-Di-*O*-methyl (1,2,5,6-tetra-*O*-acetyl)	trace	trace
2,4-Di-*O*-methyl (1,3,5,6-tetra-*O*-acetyl)	1	1
2-Mono-*O*-methyl (1,3,4,5,6 penta-*O*-acetyl)	n.d.	0.3
2-N-Methylacetamido-2-deoxyglucitol		
3,4,6-Tri-*O*-methyl (1,5-di-*O*-acetyl)	trace	0.4
3,6-di-*O*-methyl (1,4,5-tri-*O*-acetyl)	2.3	1.2
1,3,5,6-Tetra-*O*-methyl (4-*O*-acetyl)	0.4	0.3
1,3,5-Tri-*O*-methyl (4,6-di-*O*-acetyl)	0.2	0.1
6-Mono-*O*-methyl (1,3,4,5-tetra-*O*-acetyl)	n.d.	0.2

[a]Numbers were calculated by taking the value of 2,4-di-*O*-methylmannitol as 1.0.
[b]Non-quantitative recovery of these derivatives in highly branched structures has previously been reported (Yamashita *et al.* (1984).

position differences between the brain and thymus forms and we cannot exclude that this will not affect intracellular transport routes (Fatemi and Tartakoff, 1986). A major conclusion of the analysis is that rat brain Thy-1 and thymus Thy-1 do not have any glycoforms in common (Figure 7). A definition of 'Thy-1' must therefore include a set of glycoforms and on this definition brain and thymus Thy-1 are different molecules.

In the mouse, Thy-1 is present in thymocytes and mature T-cells (Morrison *et al.*, 1985), whereas in the rat it is expressed on thymocytes and not on mature T-cells. Detailed structural analysis of mouse thymocyte Thy-1 (which also fractionates into L^+ and L^- forms) has not been completed. However, partial characterization indicates that there are certain structural features in common with the rat thymus Thy-1 (Carlsson and Stigbrand, 1984). In particular, mouse thymocyte Thy-1 has been reported to contain terminal α-galactose residues (Van Halbeek *et al.*, 1985) and studies using the *Datura stramonium* lectin suggest that 50% of mouse thymocyte Thy-1 contains polylactosaminoglycan-type structures (Morrison *et al.*, 1985). In addition, the disposition of sugar chains at the individual sites is similar (i.e. most oligomannose structures are located at site 23 in mouse thymocyte Thy-1) (Morrison *et al.*, 1984; Carlsson, 1985). Glycosylation characteristics that are conserved across species may be clues to the identity of oligosaccharides which have important functional and/or structural roles (Kobata, 1984; Yamashita *et al.*, 1985; Rademacher *et al.*, 1986).

Lectin fractionation of intact glycoproteins may provide important information about the intricacies of oligosaccharide biosynthesis. Thymus Thy-1 L^+ and Thy-1 L^- were separated on the basis of structures at site 74, yet structures at sites 23 and 98 also correlated with the separation even though they were not directly involved in the binding. In particular, no polylactosaminoglycans are found in the Thy-1 L^+ population despite the fact that these structures are present at sites 23 and 98. Since these are 'passenger sites' in this lectin fractionation (i.e. most of their structures do not interact with lentil lectin), their oligosaccharide distribution on Thy-1 L^+ should reflect the oligosaccharide heterogeneity pattern of these sites on the unfractionated Thy-1. That it does not raises the possibility that the biosynthesis at a given glycosylation site is not independent of biosynthetic events occurring at other glycosylation sites — thus giving rise to only certain allowed glycoforms. Such a mechanism would be necessary if oligosaccharides on cell-surface glycoproteins are recognized in clusters and not individually. This may be the reason why the majority of glycoproteins contain multiple attachment sites. Alternatively these results may indicate an heterogeneous cellular origin of the thymus Thy-1 glycoprotein. Further experiments are necessary to distinguish between these two possibilities.

Finally the results of this study have implications for the genetic engineering of glycoproteins. If glycoforms have evolved to mediate tissue-specific differentiation, then the production of 'native' glycoproteins by genetic engineering may only be possible from its tissue (or actual cell of origin) at the appropriate stage of differentiation. In support of this is the recent finding that the developmental expression of the polylactosaminoglycans on Thy-1 during T-cell differentiation in the mouse (Morrison *et al.*, 1985) differs from the developmental expression of polylactosaminoglycans on Band 3 during human hematopoiesis (Fukuda and Fukuda, 1984). The importance of a specific oligosaccharide sequence may therefore only be appreciated in its appropriate environment.

Materials and methods

Purification of Thy-1 glycoprotein

Thy-1 from rat brain was isolated according to the method of Campbell *et al.* (1981). Rat brains were homogenized and then extracted with $CHCl_3$/MeOH (2:1 v/v). The insoluble residue was solubilized with 5% sodium deoxycholate buffer and passed through an MRC OX-7 (anti-Thy-1.1) monoclonal antibody affinity column after a bovine-γ-globulin Sepharose 4B precolumn. The bound antigen was eluted with 50 mM diethylamine HCl pH 11.5/0.5% sodium deoxycholate and neutralized with glycine. Finally Thy-1 antigen was chromatographed on a Sephacryl S-200 column in 5% sodium deoxycholate. From 1 g of rat brain 35 μg of Thy-1 were obtained. The purity and amount of the preparation was checked by SDS–PAGE, amino acid analysis and antigen inhibition assay. Thy-1 from rat thymocytes was prepared in a similar fashion to that from brain. Approximately 0.5 mg of Thy-1 was obtained per 10^{11} cells. Thymocyte Thy-1 was further resolved into two populations (L^+ and L^-) by using lentil lectin (Letarte, 1984). The L^- form did not bind. The L^+ form was eluted from the lentil lectin affinity column with 0.2 M α-methylglucopyranoside in sodium deoxycholate buffer.

Isolation and purification of glycopeptides

A total of 4% w/w trypsin was added to the completely reduced and alkylated protein in 0.1 M NH_4HCO_3 pH 8 in the presence of Brij 96 (0.5% w/v) and incubated at 37°C for 36 h. The digest was chromatographed on a Bio-Gel P-30 (1 × 150 cm) gel filtration column (thymocyte) [or on a Bio-Gel P-10 column (brain)] and pools containing glucosamine determined by amino acid analysis were

labelled A,B,C,D in descending order of hydrodynamic volume. Pool A, the void, contained undigested material and the lipid tail. The glycopeptide pools were then subjected to reverse-phase h.p.l.c. separation on a C_{18} μBondapak column (Waters). The initial and final conditions for separation of tryptic peptides were 5 and 60% acetonitrile (brain) and 3.5 and 60% (thymocyte) in 10 mM ammonium acetate. An aliquot from each glucosamine-positive pool was taken for amino acid analysis for identification of the glycosylation site of the glycopeptide, estimation of quantity and assessment of purity.

Release of asparagine-linked sugar chains

Between 10 and 150 nmol of Thy-1 glycoproteins or glycopeptides (salt-free) derived from Thy-1 (see Table I and Figure 1) were cryogenically dried over activated charcoal at −196°C ($< 10^{-6}$ bar). The Thy-1 samples were suspended in 200−300 μl of freshly distilled anhydrous hydrazine (toluene/CaO, 25°C, 10 Torr) under an anhydrous argon atmosphere. The temperature was raised 17°C/h from 30 to 85°C and then maintained at 85°C for a further 12 h. The hydrazine was removed by evaporation under reduced pressure ($< 10^{-5}$ bar) at 25°C followed by repeated (5 ×) flash evaporation from anhydrous toluene (thiophen and carbonyl free). The hydrazinolysates were N-acetylated by the addition of excess (5 × amino groups) acetic anhydride (0.5 M) in saturated $NaHCO_3$ at 4°C for 10 min. The temperature was then raised to 25°C and a second aliquot of acetic anhydride was added. The reaction was allowed to proceed for a further 50 min. Following N-acetylation, the samples were applied to a column of Dowex AG50X 12 (H^+), eluted with water, and evaporated to dryness (27°C). The desalted samples were dissolved in water and applied to Whatman 3 MM chromatography paper. Descending paper chromatography (27°C, 70% RH) was subsequently performed using n-butanol/ethanol/water (4:1:1 v/v) (solvent I). After 48 h the first 5 cm measured from the origin were eluted with water. The oligosaccharides so isolated were flash-evaporated to dryness (27°C), and reduced with 5-fold molar excess of 6 mM NaB^3H_4 (12 Ci/mmol, obtained from New England Nuclear Co.) in 50 mM NaOH adjusted to pH 11 with saturated boric acid (30°C, 4 h). An equivalent volume of 1 M $NaBD_4$ in NaOH/boric acid pH 11 was then added and the incubation continued for a further 2 h. The mixture was then acidified (pH 4−5) with 1 M acetic acid and applied to a Dowex AG50X 12 (H^+) column, eluted with water, evaporated to dryness (27°C) and flash-evaporated (27°C) from methanol (5 ×). The samples were then applied to Whatman 3 MM paper and subjected to descending paper chromatography for 2 days using solvent I.

Radiochromatogram scanning was performed with an LB230 Berthold radiochromatogram scanner. The radioactivity remaining at the origin was subsequently eluted with water. The isolated oligosaccharides were subjected to high-voltage paper electrophoresis in pyridine/acetic acid (pH 5.4, Whatmann 3 MM, 80 V/cm) in order to determine the relative proportion of neutral (N) and acidic (A) components [(lactitol (L) and 3′(6′) sialyl lactitol (SL) were used as standards]. In order to determine the chemical identity of the acidic components an aliquot of the reduced ^3H-labelled oligosaccharides so isolated were subjected to exhaustive neuraminidase digestion (*Arthrobacter ureafaciens* neuraminidase 10 units/ml in 0.1 M acetate buffer pH 5.0 for 18 h at 37°C) and the products subjected to high-voltage paper electrophoresis. All radioactivity remained at the origin following neuraminidase digestions (inserts Figure 2a, c). The neuraminidase-treated samples were recovered from paper by elution with water, desalted using a tandem column of Chelex 100 (Na^+)/Dowex AG50X 12 (H^+)/AG3X 4A (OH^-)/QAE-A25 Sephadex, eluted in water, evaporated to dryness and resuspended in 10 μl of a 20 mg/ml partial dextran hydrolysate and applied to a Bio-Gel P-4 (−400 mesh) gel permeation chromatography column (1.5 × 200 cm). The eluant was monitored for radioactivity using an LB503 Berthold h.p.l.c. radioactivity monitor and for refractive index using an Erma ERC 7510 refractometer. Analogue signals from the monitors were digitized using Nelson Analytical ADC interfaces. The digital values were collected and analysed using Hewlett−Packard 9836 C computers. The P-4 chromatograms show radioactivity (vertical axis) plotted against retention time. The numerical superscripts refer to the elution position of glucose oligomers in glucose units as detected simultaneously by the refractive index monitor (data not shown). V_0 is the void position. Sample elution positions (in glucose units) were calculated by cubic spline interpolation between the internal standard glucose oligomer positions.

Carbohydrate composition

Composition of oligosaccharides was determined by g.l.c. of the per-O-trimethylsilyl derivatives following methanolysis. Methanolysis was performed as follows. The oligosaccharide (1−5 nmol) to be analysed was mixed with a known amount of silo-inositol (Sigma) and the mixture dried in a glass capillary tube by evaporation from methanol (2 ×). Methanolic HCl (0.5 M, 50 μl) and 10 μl methyl acetate were added and incubated at 70°C for 16 h. Per-acetylation was subsequently performed by the addition of 5 μl acetic anhydride in 10 μl pyridine followed by incubation for 30 min at room temperature. The sample was then dried and the per-acetylated monosaccharides derivatized by the addition of 15 μl of Sil-A (Sigma) and incubation over CaO for 15 min at room temperature. The per-O-trimethylsilyl derivatives were analysed on a Hewlett−Packard 5996C g.l.c.−m.s. The identity of the reducing terminal monosaccharide was determined using the radio-electrophoretic method (Takasaki, 1978) omitting the reduction step and after double hydrolysis in 1 M HCl (i.e. HCl/N-acetylation/HCl/N-acetylation) of the tritium-labelled oligosaccharide.

Oligosaccharides

Standard oligosaccharides were prepared from glycoproteins by hydrazinolysis with subsequent reduction with either NaB^3H_4 (OT) or NaB^2H_4 (OD). The asialo oligosaccharides were obtained by neuraminidase digestion. Other standard oligosaccharides were obtained by exoglycosidase digestion of the oligosaccharides listed below.

(i) Galβ1 → 4GlcNAcβ1 → 2Manα1 → 6[Galβ1 → 4GlcNAcβ1 → 4(Galβ1 → 4GlcNAβ1 → 2) Manα1 → 3]Manβ1 → 4GlcNAcβ1 → $4GlcNAc_{OT}$ was prepared from bovine fetuin. (ii) GlcNAc β1 → 2Manα1 → 6[GlcNAcβ1 → 4(GlcNAcβ1 → 2)Manα1 → 3]Manβ1 → 4GlcNAcβ1 → $4GlcNAc_{OT}$ was prepared from (i) by treatment with jack bean β-galactosidase. (iii) Manα1 → 6(Manα1 → 3)Manα1 → 6(Manα1 → 3)Manβ1 → 4GlcNAcβ1 → $4GlcNAc_{OT}$ was prepared from bovine ribonuclease B. (iv) Galβ1 → 4GlcNAcβ1 → 2Manα1 → 6(Galβ1 → 4GlcNAcβ1 → 2Manα1 → 3)Manβ1 → 4GlcNAcβ1 → $4GlcNAc_{OT}$ was prepared from human serum transferrin. (v) Galβ1 → 4GlcNAcβ1 → 2Manα1 → 6(Galβ1 → 4GlcNAcβ-1 → 2Manα1 → 3)Manβ1 → 4GlcNAcβ1 → 4(Fucαl → 6)$GlcNAc_{OT}$ was prepared from human serum IgG. (vi) GlcNAcβ1 → 2Manα1 → 6(GlcNAcβ-1 → 2Manα1 → 3)Manβ1 → 4GlcNAcβ1 → $4GlcNAc_{OT}$ was prepared from (iv) by β-galactosidase treatment. (vii) GlcNAcβ1 → 2Manα1 → 6(GlcNAcβ-1 → 2Manα1 → 3)Manβ1 → 4GlcNAcβ1 → 4(Fucα1 → 6)$GlcNAc_{OT}$ was prepared from human serum IgG. (viii) Manα1 → 6(GlcNAcβ1 → 2Man1α → 3)Manβ1 → 4GlcNAcβ1 → 4(Fucα1 → 6)$GlcNAc_{OT}$ was prepared by exoglycosidase digestion of monosialylated (v). (ix) GlcNAcβ1 → 2Manα1 → 6(Manα1 → 3)Manβ1 → 4GlcNAcβ1 → 4(Fucα-1 → 6)$GlcNAc_{OT}$ was prepared by exoglycosidase digestion of monosialylated (v). (x) Manα1 → 6(Manα1 → 3)Manβ1 → 4GlcNAc β1 → 4(Fucα1 → 6) $GlcNAc_{OT}$ was obtained by β-galactosidase and jack bean β-*N*-acetylhexosaminidase digestion of (vii). (xi) Manβ1 → 4GlcNAcβ1 → 4(Fucα1 → 6)$GlcNAc_{OT}$ was obtained by jack bean α-mannosidase digestion of (x). (xii) GlcNAcβ1 → 4(Fucα1 → 6)$GlcNAc_{OT}$ was obtained from snail β-mannosidase digestion of (xi). (xiii) Fucα1 → 6$GlcNAc_{OT}$ was obtained from jack bean β-*N*acetylhexosaminidase digestion of (xii), (xiv) $GlcNAc_{OT}$ was obtained by reduction of *N*-acetylglucosamine with NaB^3H_4. (xv) GlcNAcβ1 → $4GlcNAc_{OT}$ was obtained from α-fucosidase treatment of (xii). (xvi) Galβ1 → 4(Fucα1 → 3)GlcNAcβ1 → 2Manα1 → 6(Galβ1 → 4GlcNAcβ1 → 2Manα1 → 3) Manβ1 → 4GlcNAcβ1 -4(Fucα1 -6)$GlcNAc_{OT}$ was prepared from human lactoferrin. (xvii) Galα1 → 3Galβ1 → 4GlcNAcβ1-2Manα1 → 6(3)(Galβ1-4GlcNAcβ1-2Manα1 → 3(6))Manβ1 → 4GlcNAcβ1 → 4(Fucα1 → 6)$GlcNAc_{OT}$ was prepared from bovine thyroglobulin.

Exoglycosidase digestion

Digestion of tritium-labelled oligosaccharides (~2×10^5 c.p.m.) with exoglycosidases with defined specificities (Kobata, 1984) was carried out under the following conditions: jack bean β-galactosidase, 20 μl of 6 units/ml in 0.05 M sodium citrate, pH 3.5; jack bean β-*N*-acetylhexosaminidase, 20 μl of 10 units/ml in 0.1 M citrate−phosphate, pH 4.0; jack bean α-mannosidase, 20 μl of 10 units/ml in 0.1 M sodium acetate, pH 4.5; *Streptococcus pneumoniae* β-galactosidase, 20 μl of 0.2 units/ml in 0.1 M citrate−phosphate, pH 6.0; *S. pneumoniae* β-*N*-acetylhexosaminidase, 20 μl of 0.1 units/ml in 0.1 M citrate−phosphate, pH 6.0; *Aspergillus phoenicis* α-mannosidase, 20 μl of 20 μg/ml in 0.1 M sodium acetate, pH 5.0; *Charonia lampas* α-fucosidase, 20 μl of 3 units/ml in 0.1 M sodium acetate/0.2 M NaCl, pH 6.0; bovine epididymis α-fucosidase, 20 μl of 1 unit/ml in 0.2 M citrate phosphate, pH 6.0; *Arthrobacter ureafaciens* neuraminidase, 20 μl of 10 units/ml 0.1 M sodium acetate, pH 5.0; coffee bean α-galactosidase, 20 μl of 5 units/ml in 0.1 M sodium acetate, pH 5.5; *Achatina fulica* β-mannosidase, 20 μl of 0.2 units/ml in 0.5 M sodium citrate, pH 4.0; *Bacillus fulminans* α-fucosidase, 10 μl of 0.5 mg/ml in 0.1 M citrate−phosphate, pH 6; *Streptomyces plicatus* endoglycosidase H, 10 μl of 3 μg/ml in 0.1 M sodium citrate, pH 5.5;*Escherichia freundii* endo-β-galactosidase, 10 μl of 0.5 units/ml in 0.1 M sodium acetate, pH 5.8; bovine kidney α-fucosidase, 10 μl of 3 units/ml in 0.2 M sodium acetate, pH 4.5; bovine kidney β-*N*-acetylhexosaminidase, 10 μl of 3 units/ml in 0.2 M sodium acetate, pH 4.5; *Tritrichomonas foetus* mix, 10 μl of 10 mg/ml in 0.1 M sodium acetate, pH 5.0. Where applicable, 1 unit of glycosidase was defined as the amount of enzyme that, in control experiments, released 1 μmol of 4-nitrophenol, from the respective 4-nitrophenyl glycoside, per minute at 37°C. All incubations were carried out for 18 h at 37°C under toluene and were terminated by heating at 100°C for 2 min.

Methylation analysis

Reduced oligosaccharides (1–10 nmol) were dissolved in 50 μl dimethyl sulphoxide. A further 50 μl of 120 mg/ml colloidal solution of NaOH in dimethyl sulphoxide was added and the mixture incubated at room temperature with stirring for 10 min. Aliquots of methyl iodide (10 μl) (Fluka) were added, the first after 15 min, the second after 20 min and the third after 25 min. The partially methylated oligosaccharides were extracted into the organic phase by the addition of 0.3 ml chloroform and 1 ml of a 100 mg/ml solution of sodium thiosulphate, with thorough mixing. The aqueous phase was discarded and the organic phase extracted four times with 4 × 1 ml of sodium thiosulphate solution. The organic phase was evaporated to dryness under reduced pressure and 0.1 ml of 93% acetic acid/0.25 N sulphuric acid added (80°C for 2.5 h). The sample was then passaged through an AG3X 4A (acetate) column (bed volume 0.5 ml) and the column washed five times with 0.5 ml of 50% methanol (v/v). The sample and washings were evaporated to dryness and the partially methylated monosaccharides reduced by the addition of 0.2 ml of 10 mg/ml NaB^2H_4. The sample was then neutralized with acetic acid and borate removed by flash-evaporation (5 ×) from acidified methanol (0.3 ml). Acetic anhydride (0.25 ml) was then added and the sample incubated at 80°C for 3 h. The acetic anhydride was removed by evaporation under vacuum and the partially methylated alditol acetates (PMAA) extracted by addition of 0.5 ml dichloromethane and 1 ml water. After thorough mixing the aqueous phase was discarded. Analysis of the PMAAs was performed on a Hewlett–Packard 5996C g.l.c–m.s. system fitted with on-column injection and flame ionization detection. Separation was by capillary g.l.c. on both bonded OV17 (0.32 mm × 50 m, Alltech Associates, Carnforth, Lancs, UK) and SP1000 (0.25 mm × 50 m, Supelco, R.B. Radley and Co. Ltd, Sawbridgeworth, Herts, UK) stationary phases. The temperature programmes were 90–200°C at 20°C/min and 90–240°C at 10°C/min, for OV17 and SP1000 respectively.

Identification was based on retention time and mass spectrum by comparison with synthetic reference compounds or published data. Quantitation was carried out by calculation from the flame ionization detector response using the effective carbon response value or the molar response measured for the authentic compound.

Analysis of oligosaccharides on intact Thy-1 and on glycopeptides

Where feasible, the frequency of an oligosaccharide characteristic was determined by selective fractionations of the entire oligosaccharide pool (see Figure 4 for examples). (This strategy results in increased accuracy compared with the summation of the molar proportion of each individual oligosaccharide.) The neutral (N) versus acidic (A) ratios were obtained by high-voltage paper electrophoresis as described in the legend to Figure 2. The incidence of complex-type, lactosaminoglycan, oligomannose-type, hybrid-type, core fucose and non-reducing terminal galα →, either in the neutral or acidic population, was determined by using endo- and exoglycosidases either uniquely or in mixtures so as to produce digestion products which, when analysed by Bio-Gel P-4 chromatography, were diagnostic for each of the individual classes or substitutions (Kobata, 1979, 1984).

Oligomannose. The occurrence of these structures was determined by incubation of an aliquot of neuraminidase-treated oligosaccharides (asialo) with jack bean α-mannosidase: 1 unit in 10 μl of 0.1 M sodium acetate, pH 5.0 (Yamashita *et al.*, 1980a). The digestion product $(Man\beta1\text{-}4GlcNAc\beta1\text{-}4GlcNAc)_{OT}$, which elutes from a Bio-Gel P-4 column at 5.5 glucose units (gu), is diagnostic for the presence of oligomannose structures. Sequential enzymatic digestion of individual P-4 fractions using α(1,2)-specific mannosidase from *A. phoenicis* (Yamashita *et al.*, 1980b), jack bean α-mannosidase, snail β-mannosidase and jack bean β-*N*-acetylhexosaminidase (Li and Li, 1972) confirmed that the oligomannose structures original present were only $Man_6GlcNAc_2$ and $Man_5GlcNAc_2$ (9.8 and 8.9 gu respectively) (see Figure 2b, d).

Fucose. The occurrence of core α(1,6)-linked fucose residues was determined by incubating an asialo oligosaccharide fraction with an exoglycosidase mix consisting of coffee bean α-galactosidase (4 units/ml) (Spiro and Bhoyroo; 1984), jack bean β-galactosidase (6 units/ml) (Li and Li, 1972), jack bean β-*N*-acetylhexosaminidase (10 units/ml), and jack bean α-mannosidase (100 units/ml) in 0.1 M acetate, pH 4.5. The digestion products $Man\beta1\text{-}4GlcNAc\beta1\text{-}4GlcNAc_{OT}$ (5.5 gu) and $Man\beta1\text{-}4GlcNAc\beta1\text{-}4\ Fuc(\alpha1-6)GlcNAc_{OT}$ (6.5 gu) (see Figure 4d) were diagnostic for the presence of non-fucosylated and core-fucosylated structures respectively.

Hybrid. The percentage of this oligosaccharide class was determined by incubating structures which do not reduce to $Man_5GlcNAc_2$ (8.9 gu) after *A. phoenicis* α-mannosidase digestion with *Streptomyces plicatus* endoglycosidase H (Tai *et al.*, 1975). The amount of radioactivity eluting at 2.5 gu (or 3.5 in the case of core fucosylated hybrids) was directly proportional to the occurrence of hybrid structures.

Complex. Oligosaccharide structures resistant to jack bean α-mannosidase and endoglycosidase H were sequenced individually by sequential enzymatic digestion using, either uniquely or as mixtures, the following enzymes: coffee bean α-galactosidase, jack bean β-galactosidase, jack bean β-*N*-acetyl-hexosaminidase, jack bean α-mannosidase, snail β-mannosidase, bovine epididymis α-fucosidase, *S. pneumoniae* β-N-acetylhexosaminidase (Yamashita *et al.*, 1981b) and endo-β-galactosidase from *E. freundii* (Scudder *et al.*, 1984).

Polylactosaminoglycan. Structures were classified as lactosaminoglycan if they contained $(Gal\beta1 \rightarrow 4GlcNAc\beta1 \rightarrow 3)_n$ units (Fukuda and Fukuda, 1984). The percentage of this oligosaccharide class was determined by susceptibility to endo-β-galactosidase from *E. freundii* (Scudder *et al.*, 1984).

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