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Partial characterization of the oligosaccharides of mouse thymocyte Thy-1 glycoprotein

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Four glycopeptides (I, IIA, IIB, III) with different oligosaccharide structures were isolated from purified mouse thymocyte Thy-1 glycoprotein. The glycoprotein was digested with Pronase, and the glycopeptide fraction was isolated by gel filtration and acetylated with [^{3}H]acetic anhydride. The different glycan structures were separated by affinity chromatography on concanavalin A–Sepharose 4B and lentil lectin–Sepharose 4B. Size determinations of intact and exoglycosidase- and endoglycosidase-digested glycopeptides were performed by gel filtration on Bio-Gel P-6, calibrated with glycopeptides of known structure. On the basis of these experiments and on the behaviour of the glycopeptides on the lectin columns, the following structures of the oligosaccharide chains were proposed: I, triantennary 'complex-type' with terminal fucose; IIA, biantennary 'complex-type' without fucose; IIB, biantennary 'complex-type' with fucose; III, a mixture of 'high-mannose' chains containing either five or six mannose residues (approx. 50% of each). Amino acid analysis of the glycopeptides showed that the predominant oligosaccharide at glycosylation-site Asn-23 was of 'high-mannose' type, whereas the other two sites (Asn-75 and Asn-99) were glycosylated with 'complex-type' chains. Both these sites were shown to be variably glycosylated. The major glycans linked to Asn-75 were of structures I and IIB, whereas all three 'complex-type' chains were represented at Asn-99. The results presented explain the previously reported carbohydrate heterogeneity of thymocyte Thy-1 glycoprotein.

In order to establish the specific roles of glycoproteins at cell surfaces, information on the chemical structures is needed. Hitherto, very few of the membrane-bound molecules on the lymphocyte surface have been purified and characterized, and data on the carbohydrate structures of these glycoproteins are rudimentary. Biochemical studies on the surface molecules of cells participating in the immune system will certainly facilitate the understanding of many observed phenomena in immunology, involving interactions between cells, as well as reactions between soluble factors and membrane-bound receptors.

The Thy-1 glycoprotein is expressed in relatively large amounts on the surface of thymocytes and peripheral T-lymphocytes of the mouse (Raff, 1971; Carlsson & Stigbrand, 1983*a*). Thy-1 is also present on rat thymocytes, but disappears when the cells of this species mature into immunocompetent T-lymphocytes (Acton *et al.*, 1974). The Thy-1 gene is furthermore expressed in brain tissue of many species (Dalchau & Fabre, 1979; Williams & Gagnon, 1982). The amino acid sequences of rat and mouse Thy-1 are known, and consist of 111 and 112 residues respectively (Campbell *et al.*, 1981; Williams & Gagnon, 1982). More recently a complementary-DNA clone encoding the rat thymocyte Thy-1 polypeptide was isolated and the nucleotide sequence was determined (Moriuchi *et al.*, 1983).

The glycan structures of Thy-1 have been less well characterized. The carbohydrate content is about 30% (w/w) in most species; lymphocyte Thy-1 seems to contain slightly more sugar than does the brain counterpart (Barclay *et al.*, 1976; Carlsson & Stigbrand, 1983*b*). Previous studies have shown that the carbohydrate structure of thymocyte Thy-1 is heterogeneous (Carlsson & Stigbrand, 1982). The sialic acid content varies from one to six or seven residues, and the Thy-1

Abbreviation used: GP, glycopeptide.

molecules can be separated into fractions that differ in their affinity to certain lectins (i.e. lentil lectin and wheat-germ agglutinin). The latter property was shown to be unrelated to the degree of sialylation. The heterogeneity is clearly seen when Thy-1 is analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, where it appears as a broad band (Carlsson & Stigbrand, 1982). This band was shown to be composed of several distinct species with different molecular masses (Carlsson & Stigbrand, 1983*b*). When other lymphoid cells were investigated, such as peripheral T-lymphocytes and T-lymphomas, the glycosylation pattern of Thy-1 was shown to be different (Carlsson & Stigbrand, 1983*a*). T-lymphocyte Thy-1 was more acidic, indicating a higher content of sialic acid, and the heterogeneity in the affinity to lentin lectin was altered. These results showed that the glycosylation of Thy-1 changes during maturation of thymocytes into immunocompetent T-lymphocytes.

In the present work we have investigated the structures of the oligosaccharide chains of thymocyte Thy-1, in order to evaluate the previously described carbohydrate heterogeneity and to establish the basis for studies on the differentiation-dependent glycosylation of Thy-1 in the immune system.

Materials and methods

Materials

Fetuin (type IV), human transferrin, bovine immunoglobulin G, ovalbumin (grade VI), neuraminidase (*Clostridium perfringens*, type X) and *β-N*-acetylglucosaminidase (jack bean) were obtained from Sigma Chemical Co. *β*-Galactosidase, *β-N*-acetylhexosaminidase, α-L-fucosidase (all from *Charonia lampas*) and endo-*β-N*-acetylglucosaminidase D (*Diplococcus pneumoniae*) were from Seikagaku Kogyo Co. Sephadex G-25 (fine grade), Sepharose 4B coupled with concanavalin A (10mg/ml of gel) and lentil lectin (2mg/ml of gel) were from Pharmacia Fine Chemicals. Bio-Gel P-6 (200–400 mesh) and AG-50W-X2 were from Bio-Rad Laboratories. Pronase-CB (*Streptomyces griseus*) was from Calbiochem, [^{3}H]acetic anhydride (500mCi/mmol) was from Amersham International, NaB^3H_4 (350mCi/mmol) was from New England Nuclear and endo-*β-N*-acetylglucosaminidase H (*Streptomyces griseus*) was from Miles Laboratories. Thy-1 glycoprotein was purified from mouse strain NMRI thymi as described previously (Carlsson & Stigbrand, 1983*b*).

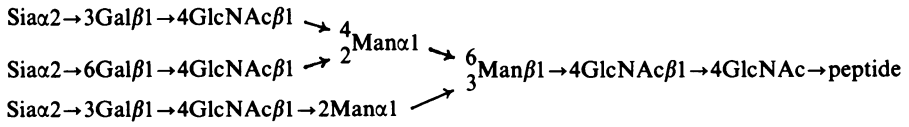
Preparation and labelling of glycopeptides

The amounts of the various glycoproteins used for glycopeptide preparation were: Thy-1, 0.8mg (45nmol); fetuin, 5mg (110nmol); transferrin, 20mg (220nmol); bovine immunoglobulin G, 10mg (70nmol); ovalbumin, 50mg (1.2μmol). The glycoproteins were dissolved in 0.5ml of 0.1M-Tris/HCl buffer, pH7.8, containing 1mM-$CaCl_2$. Pronase (1%, w/w) was added together with a small volume of toluene, and the mixtures were incubated at 60°C for 48h. Additional Pronase (1%, w/w) was added after 24h. The digestions were terminated by boiling for 3min, and the samples were gel-filtered in 0.1M-NH_4HCO_3 on a column (1cm × 100cm) of Bio-Gel P-6 (200–400 mesh) at a flow rate of 6ml/h. Fractions of volume 2ml were collected, and 100μl portions were assayed for hexose content by the phenol/H_2SO_4 method scaled down 10-fold (Hodge & Hofreiter, 1962). The hexose-positive fractions were pooled and freeze-dried. The Pronase digestion was repeated once, and the hexose-positive eluate from the second gel filtration was analysed for amino acid content and freeze-dried. The ovalbumin glycopeptides were further separated by ion-exchange chromatography, according to the procedure of Huang *et al.* (1970), on a column (1cm × 120cm) with AG-50W-X2 cation-exchange resin equilibrated and eluted with 1mM-sodium acetate buffer, pH2.6. The flow rate was 20ml/h. The peaks corresponding to GP III, GP IV and GP V (Tai *et al.*, 1975*a*) were collected and subjected to gel filtration on Bio-Gel P-6 and freeze-dried. The identity of the ovalbumin glycopeptides was confirmed by determination of the molar proportions of asparagine, hexose and glucosamine (the latter sugar was measured in the amino acid analyser), and by digestions with endoglycosidases D and H.

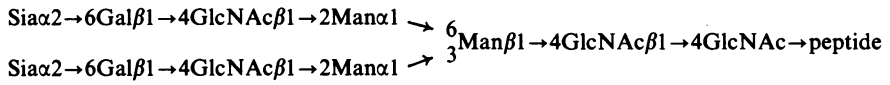
The isolated glycopeptides contained the following amounts of amino acids per mol of asparagine: fetuin GP, 0.33mol of serine; transferrin GP, 0.36mol of serine, 0.36mol of valine and 0.34mol of lysine; bovine immunoglobulin G GP,0.63mol of threonine, 0.89mol of serine and 0.21mol of glutamine. Ovalbumin glycopeptides contained asparagine as the only detectable amino acid. The amino acid composition of the Thy-1 glycopeptides is shown in the Results section. The carbohydrate structures of the previously investigated glycans are shown in Fig. 1.

Samples of the glycopeptides (20–70nmol) were each dissolved in 100μl of water to which 20μl of 1M-$NaHCO_3$ and 250μCi (500nmol) of [^{3}H]acetic anhydride in 2.5μl of toluene were added. After incubation for 30min at 20°C, the mixture was desalted on a column (1cm × 28cm) of Sephadex G-25 (fine grade) equilibrated with 0.1M-NH_4HCO_3. The radioactive glycopeptide fraction was collected and freeze-dried. The radioactivity yield was in all cases greater than 80% of the

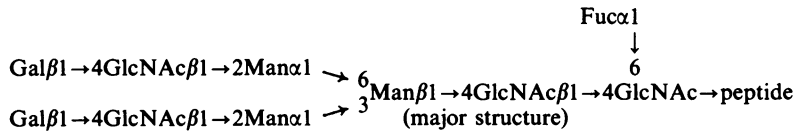
Fetuin GP



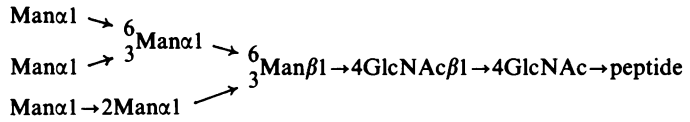
Transferrin GP



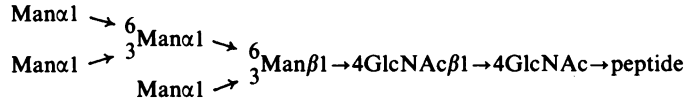
Bovine immunoglobulin G GP



Ovalbumin GP IV



Ovalbumin GP V



Ovalbumin GP III

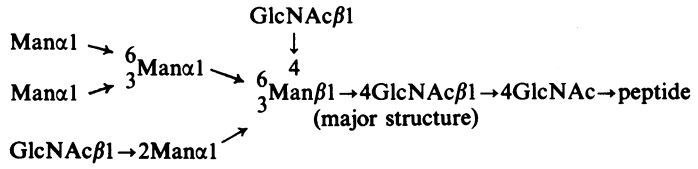


Fig. 1. *Glycan structures of marker glycopeptides used in the present study*
The structures were taken from Baenziger & Fiete (1979*a*), Spik *et al.* (1975), Tai *et al.* (1975*b*), Tai *et al.* (1977*a*) and Tai *et al.* (1975*a*).

theoretical value, indicating that the reaction was practically complete.

Preparation of oligosaccharide alcohols

The glycopeptides (1 nmol) were dissolved in 20 μl of water and the pH was adjusted to 5.5 with 10 μl of 0.1 M-sodium citrate buffer, pH 5.5. Endo-β-*N*-acetylglucosaminidase H (5 munits in 50 μl of water) was added, and the mixture was incubated at 37°C for 16 h. The digestion was terminated by boiling for 3 min, after which pH was adjusted to 12 by the addition of 50 μl of 0.05 M-NaOH. The released oligosaccharides were reduced by adding 100 μCi (300 nmol) of NaB^3H_4 in 10 μl of 0.01 M-NaOH, and the mixture was incubated at 30°C for 4 h. The reaction was terminated by adding 100 μl of 1 M-acetic acid, and the sample was freeze-dried. The labelled oligosaccharide alcohols were finally isolated on a column (0.5 ml) of concanavalin A–Sepharose (see below) and desalted.

Affinity chromatography on lectin columns

Separation of the glycan structures on concanavalin A–Sepharose columns was performed essentially as described by Finne & Krusius (1982). The glycopeptides (10nmol) were dissolved in 5mM-sodium acetate buffer, pH5.2, containing 1mM-$CaCl_2$ and 1mM-$MnCl_2$, and applied to a column of concanavalin A–Sepharose (2ml) equilibrated with the same buffer. The column was washed with 15ml of 0.1M-NaCl in equilibration buffer, after which the weakly bound glycopeptides were eluted with 15ml of 3mM-methyl α-D-glucoside and 0.1M-NaCl in equilibration buffer. The glycopeptides with high affinity for the lectin were finally eluted with 10ml of 0.5M-methyl α-D-mannoside, 1M-ethylene glycol and 0.1M-NaCl in equilibration buffer.

Lentil lectin–Sepharose columns (2ml) were equilibrated with 10mM-Tris/HCl buffer, pH8.0, containing 0.15M-NaCl, 1mM-$CaCl_2$ and 1mM-$MnCl_2$. After application of the sample (1–5nmol), the gels were washed with 10ml of equilibration buffer and elution was performed with 10ml of equilibration buffer to which 0.5M-methyl α-D-mannoside had been added.

Both lectin chromatographies were performed with a flow rate of 5ml/h, and 1ml fractions were collected. The pooled fractions were desalted on Sephadex G-25 (fine grade) before further manipulation.

Glycosidase digestions

Glycopeptides (1–5nmol) were digested at 37°C in a total volume of 100–300μl with neuraminidase (0.75units/ml) in 0.1M-sodium acetate buffer, pH4.5, for 24h, β-galactosidase (50munits/ml) in 0.2M-NaCl/0.1M-sodium acetate buffer, pH4.0, for 24h, jack-bean β-*N*-acetylglucosaminidase (1.7units/ml) in 0.1M-sodium citrate buffer, pH4.5, for 48h with further addition of enzyme (250munits) after 24h, *Charonia lampas* β-*N*-acetylhexosaminidase (50munits/ml) in 0.2M NaCl/0.1M-sodium acetate buffer, pH4.0, for 48h, α-L-fucosidase (70munits/ml) in 0.2M-NaCl/0.1M-sodium acetate buffer, pH4.0, for 48h with further addition of enzyme (10munits) after 24h, endo-β-acetylglucosaminidase D (35munits/ml) in 0.15M-sodium phosphate buffer, pH6.5, for 16h and endo-β-*N*-acetylglucosaminidase H (35munits/ml) in 0.1M-sodium citrate buffer, pH5.5, for 16h. As bacteriostatic agent a small volume of toluene was added to all incubations. The digestions were terminated by boiling for 3min.

Analytical gel filtrations

The products of the glycosidase digestions were analysed by gel filtration on a column (1cm × 100cm) of Bio-Gel P-6 (200–400 mesh) equilibrated with 0.1M-NH_4HCO_3. Samples in 1ml were applied to the column, and the flow rate was 6ml/h. Fractions of volume 1ml were collected, and 50μl portions were mixed with 2ml of Aquasol (New England Nuclear) and counted for radioactivity in an LKB–Wallac Rack-Beta scintillation counter.

The column was regularly calibrated with a mixture of [^{3}H]acetylated transferrin GP, bovine immunoglobulin G GP, ovalbumin GP IV and GlcNAc-Asn. The last-mentioned compound was prepared by endoglycosidase digestion of ovalbumin GP IV. The three former glycopeptides, together with fetuin GP, gave a straight line when the ratio between the elution volume of the respective component and the void volume (V_e/V_0) was plotted against the logarithm of the calculated M_r values. This standard curve was used to determine the apparent M_r values of the glycopeptides investigated.

Amino acid analysis

Samples (1–10nmol) were analysed for amino acid content on a Beckman 121 M amino acid analyser after hydrolysis for 24h in 5.7M-HCl at 110°C. Corrections were made for the destruction of serine and threonine, which was 11% and 5% respectively.

Results

Preparation and isolation of Thy-1 glycopeptides

Purified mouse thymocyte Thy-1 can for practical reasons only be obtained in very limited amounts. Therefore, the strategy for studying its glycosylation was to introduce a radioactive label into the peptide moieties to which carbohydrate was linked, in order to facilitate detection of the isolated structures. The peptides were made as small as possible by repeated Pronase digestions. After each digestion the glycopeptides were isolated by gel filtration. The *N*-terminals were subsequently labelled by acetylation with [^{3}H]acetic anhydride. Gel filtration of labelled glycopeptides with known glycan structures, which were used as markers, and Thy-1 glycopeptides is shown in Figs. 2(*a*) and 2(*b*). As can be seen, the Thy-1 glycopeptides were composed of a mixture of structures with different molecular masses. This mixture was separated on the basis of the glycan structure by use of lectins.

As shown by others (Krusius *et al.*, 1976), *N*-linked glycopeptides can be separated into three fractions on concanavalin A–Sepharose, on the basis of different affinities for the lectin. The prerequisite for binding has been shown to be the presence of free hydroxy groups at C-4, C-6 and probably also at C-3 on the mannose residues α-

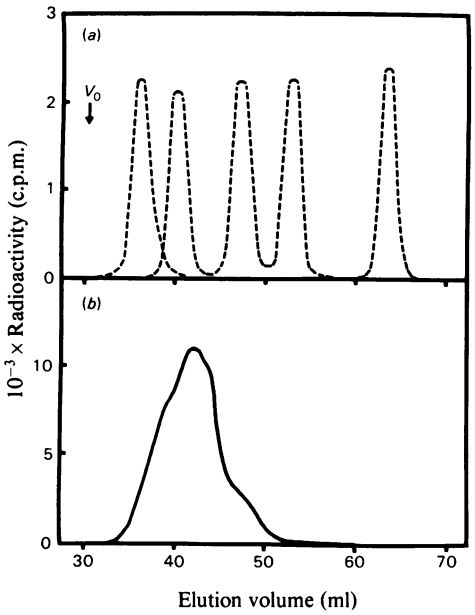


Fig. 2. *Gel filtration of [^{3}H]acetylated glycopeptides* Purified glycoproteins were digested with Pronase, and the glycopeptides were isolated and labelled with [^{3}H]acetic anhydride and subjected to gel filtration on Bio-Gel P-6. (*a*) Molecular-mass markers (from left to right): fetuin GP (M_r 3050), transferrin GP (M_r 2500), bovine immunoglobulin G GP (M_r 1900), ovalbumin GP IV (M_r 1510) and GlcNAc-Asn (M_r 355). V_0 was determined with bovine serum albumin. Free mannose was eluted at 84ml. (*b*) Glycopeptides from thymocyte Thy-1. Fractions of volume 1ml were collected and radioactivity was measured on 50μl samples.

linked to the β-linked core mannose (Baenziger & Fiete, 1979*b*). In addition, the hydroxy group at C-4 on the β-linked mannose has to be free. If any of these hydroxy groups are blocked, as is the case for tri- and tetra-antennary 'complex-type' chains, the binding is impaired and the glycopeptide will pass unretarded through the column. The common structure of biantennary 'complex-type' chains, as represented by transferrin oligosaccharide, has *N*-acetylglucosamine residues linked β-(1→2) to the α-linked mannose residues. These structures consequently bind to concanavalin A but can be eluted with low concentrations of competing sugar. If, however, the glycans contain additional mannose residues (i.e. 'high-mannose' chains) the binding will be stronger, and higher concentrations of sugar are needed for elution.

The marker glycopeptides were used to establish the conditions for good separation of the three types of glycan structures on a concanavalin A–Sepharose column. By the use of 3mM-methyl α-D-glucoside and 500mM-methyl α-D-mannoside/1M-ethylene glycol as eluting agents, a mixture of fetuin GP, transferrin GP and ovalbumin GP IV was completely separated into its components (results not shown). The same conditions were used for Thy-1 glycopeptides, and the result is shown in Fig. 3. Three fractions were obtained, i.e. GP I, GP II and GP III, in the relative amounts 27%, 42% and 31% respectively, as determined from the radioactivity measurements.

It has previously been shown that, when Thy-1 molecules from mouse or rat thymocytes are applied on a column of lentil lectin–Sepharose, about half of the material is bound, whereas the

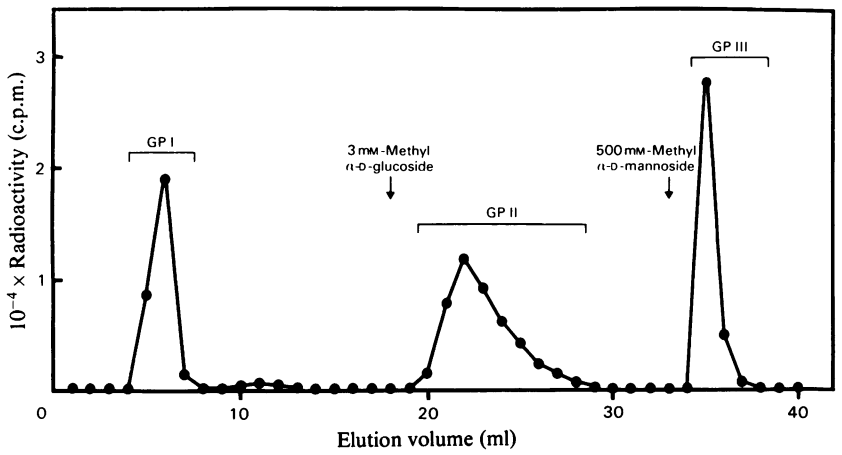


Fig. 3. *Isolation of different oligosaccharide structures from thymocyte Thy-1 on concanavalin A–Sepharose* ^{3}H-labelled glycopeptides were applied to a concanavalin A–Sepharose column (2ml). After a washing, two fractions with different affinities to the lectin were eluted by use of different concentrations of the sugars indicated. Fractions were pooled as indicated by the bars.

other half passes through the column unbound (Carlsson & Stigbrand, 1982: Letarte-Muirhead *et al.*, 1975). The latter fraction has a slightly larger molecular mass, which indicates the presence of a blocking residue, impairing the binding to the lectin (Barclay *et al.*, 1976; Carlsson & Stigbrand, 1983*b*). The isolated glycopeptides were tested for binding to lentil lectin in attempts to separate the glycan structures further. As shown in Figs. 4(*a*) and 4(*c*), neither GP I nor GP III was bound to the column. However, GP II could be separated into two fractions (i.e. GP IIA and GP IIB) with different affinities for the lectin (Fig. 4*b*). The distribution of unbound and bound glycopeptide was 52% and 48% respectively.

The structural requirements for binding to lentil lectin have been studied in detail (Kornfeld *et al.*, 1981). As is also the case with concanavalin A, the hydroxy groups on C-4 on the α-linked mannose residues have to be unblocked for binding to lentil lectin. In addition, a fucose residue linked to the innermost *N*-acetylglucosamine residue has been shown to be necessary. In agreement with these findings, the only marker glycopeptide that was able to bind to lentil lectin–Sepharose was bovine immunoglobulin G GP (results not shown). When Thy-1 GP IIB (Fig. 4*d*) and bovine immunoglobulin G GP (not shown) were digested with α-L-fucosidase and tested on a lentil lectin–Sepharose column, the major part of the glycopeptide material passed through the column unbound. This confirms the importance of a fucose residue for binding to the lectin. The small amount that still bound to the lectin was probably the result of incomplete digestion.

The lectin-binding experiments indicate that thymocyte Thy-1 contains different 'classes' of *N*-linked glycans, i.e. tri- or tetra-antennary 'complex-type' chains (GP I), biantennary 'complex-type' chains with (GP IIB) and without (GP IIA) fucose, and 'high-mannose' chains (GP III). In order to investigate these structures further, digestions with exo- and endo-glycosidases and size determinations by gel filtration were performed.

Size determinations and glycosidase digestions of isolated glycopeptide structures

The isolated structures GP I, GP IIA and GP IIB were subjected to gel filtration on a Bio-Gel P-6 column and the elution patterns were compared with that of the marker glycopeptides [Figs. 5(*a*)–5(*c*)]. The major material of GP I was eluted between fetuin GP and transferrin GP, whereas GP IIA and GP IIB were eluted somewhat later than transferrin GP. As previously shown (Carlsson & Stigbrand, 1982), thymocyte Thy-1 is heterogeneous with respect to the sialic acid content, and it seems reasonable to assume that only a small fraction of the glycopeptides is fully sialylated. Therefore the migrations obtained are to be expected for triantennary and biantennary structures respectively that are lacking one or a few

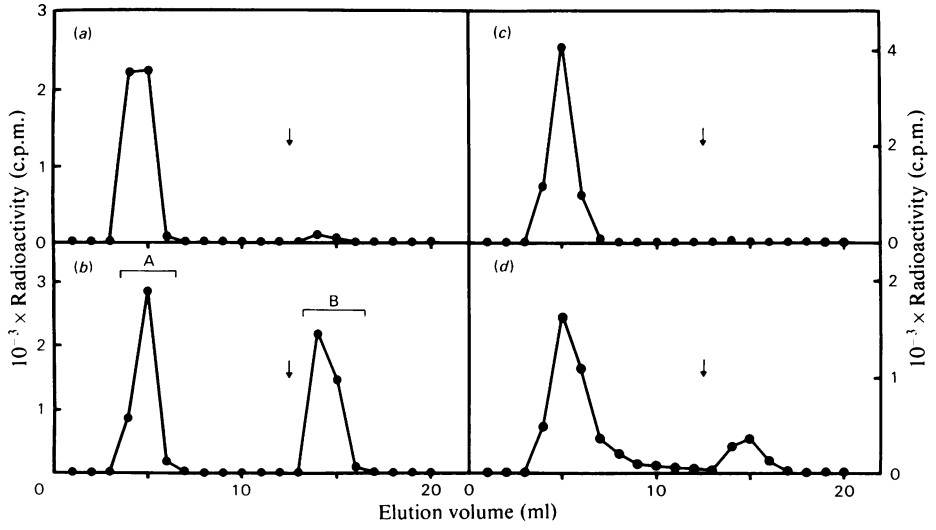


Fig. 4. *Affinity chromatography of Thy-1 glycopeptides on lentil lectin–Sepharose*
Glycopeptide fractions isolated on concanavalin A–Sepharose were applied to lentil lectin–Sepharose columns (2ml). After a washing, 500mM-methyl α-D-mannoside was applied as indicated by the arrows. (*a*) GP I; (*b*) GP II; (*c*) GP III. Glycopeptide fractions GP IIA and GP IIB were pooled as indicated by the bars. (*d*) A portion of GP IIB was treated with α-L-fucosidase and applied to the same column.

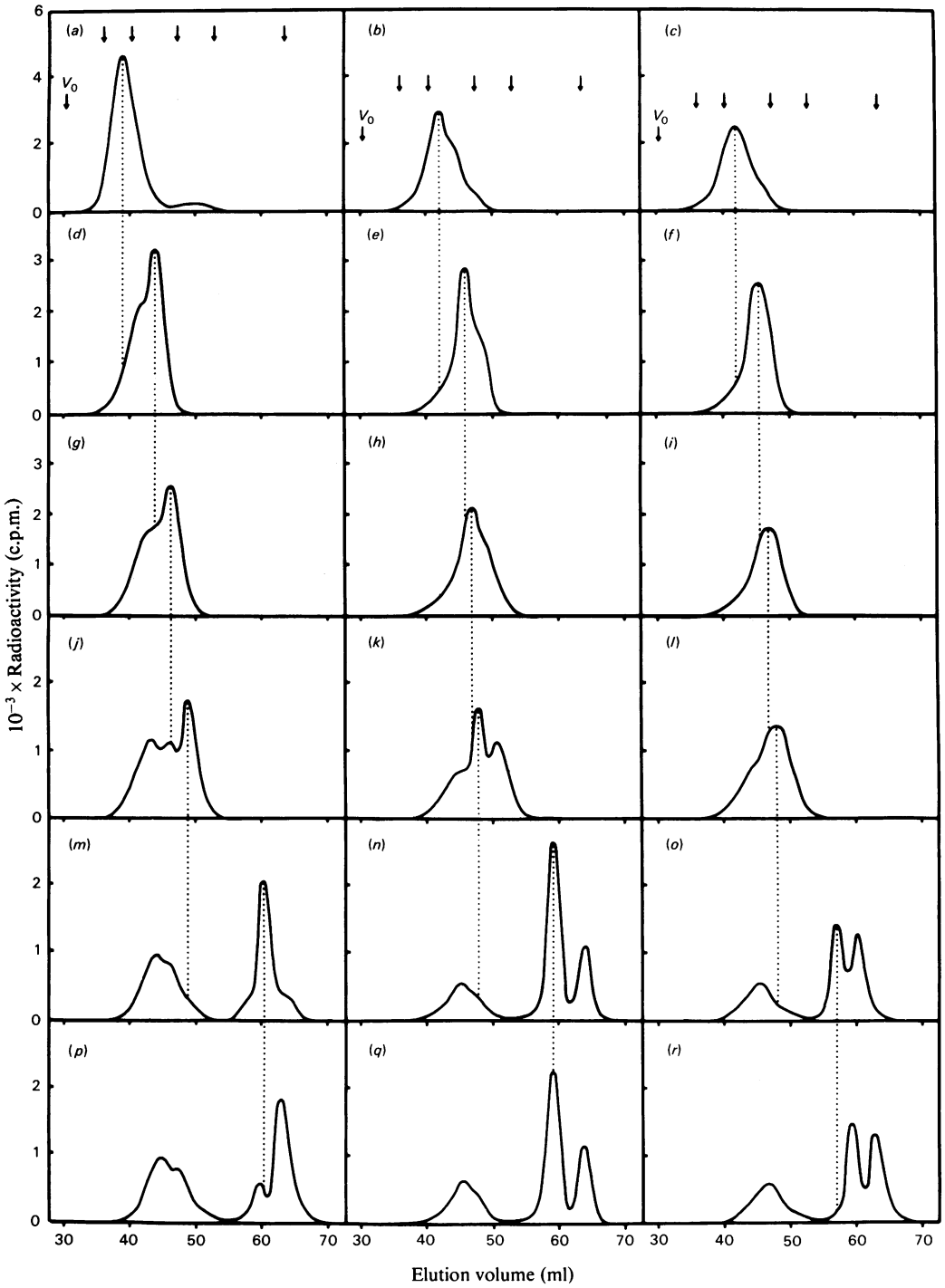


Fig. 5. *Gel filtration of intact and stepwise-glycosidase-treated Thy-1 glycopeptides I, IIA and IIB*

Glycopeptides (1 ml) were applied to a column of Bio-Gel P-6. Fractions of volume 1 ml were collected and radioactivity was measured on 50 μl samples. The arrows denote elution of the molecular-mass markers shown in Fig. 2(*a*). (*a*), (*d*), (*g*), (*j*), (*m*) and (*p*) GP I; (*b*), (*e*), (*h*), (*k*), (*n*) and (*q*) GP IIA; (*c*), (*f*), (*i*), (*l*), (*o*) and (*r*) GP IIB. (*a*)–(*c*) Untreated glycopeptides; (*d*)–(*r*) glycopeptides treated with (*d*)–(*f*) neuraminidase, (*g*)–(*i*) β-galactosidase, (*j*)–(*l*) β-*N*-acetylhexosaminidase, (*m*)–(*o*) endo-β-*N*-acetylglucosaminidase D and (*p*)–(*r*) α-L-fucosidase. The vertical dotted lines show the elution of the major peak of each glycopeptide fraction.

sialic acid residues when compared with structures of the same type that have all galactose residues occupied by sialic acid. The sialic acid heterogeneity may also in part be responsible for the rather broad peaks obtained.

The Thy-1 and marker glycopeptides were subjected to sequential digestions with exoglycosidases (i.e. neuraminidase, β-galactosidase and β-*N*-acetylglucosaminidase). By use of the marker glycopeptides, the apparent molecular-mass decrease after removal of the respective monosaccharide was measured. The values obtained were 260Da and 275Da per sialic acid residue, 60Da and 65Da per galactose residue and 155Da and 145Da per *N*-acetylglucosamine residue for fetuin GP and transferrin GP respectively. When the Thy-1 glycopeptides were treated with neuraminidase [Figs. 5(*d*)–5(*f*)], β-galactosidase [Figs. 5(*g*)–5(*i*)] and β-*N*-acetylglucosaminidase [Figs. 5(*j*)–5(*l*)], the apparent molecular-mass decrease of the major peaks corresponded to 1.8, 1.2 and 1.1 sialic acid residues, 2.8, 1.9 and 1.8 galactose residues, and 1.7, 0.7 and 1.2 *N*-acetylglucosamine residues for GP I, GP IIA and GP IIB respectively, as compared with the marker glycopeptides subjected to the same treatment. It is therefore further supported that GP I has a triantennary structure and GP IIA and GP IIB are of the biantennary type, and that the antennae are composed of the ordinary (sialic acid→)galactose→*N*-acetylglucosamine sequence. The removal of the *N*-acetylglucosamine residues from the Thy-1 glycopeptides seemed to be incomplete. Approximately one residue per glycopeptide appeared to be insensitive to β-*N*-acetylglucosaminidase digestion. Even after repeated treatment with enzymes from different sources, no further decrease in size was obtained. The reason for this phenomenon is unknown, but it has also been shown for similar glycans by others (Baenziger & Fiete, 1979*a*). One possibility is that the action of the glycosidases is influenced by the peptide moiety.

In ordinary *N*-linked 'complex-type' glycan structures the *N*-acetylglucosamine residues of the branches are β-linked to mannose residues that in turn are α-linked to the β-linked core mannose (Sharon & Lis, 1982). If the mannose bound through an α-(1→3)-linkage to the core mannose is exposed terminally, the enzyme endo-β-*N*-acetylglucosaminidase D is able to cleave the glycan between the core *N*-acetylglucosamine residues, leaving only one or two sugar residues attached to the peptide (Tai *et al.*, 1975*a*). When the exoglycosidase-treated Thy-1 glycopeptides were digested with this enzyme, the major peaks decreased further in size [Figs. 5(*m*)–5(*o*)]. The molecular-mass decrease was slightly larger than that obtained with exoglycosidase-digested fetuin GP and transferrin GP subjected to the same treatment (results not shown), which favours the suggestion that the Thy-1 glycopeptides contained indigestible *N*-acetylglucosamine residues. These residues could not, however, belong to the α-(1→3)-linked branch, since most of the material in the major peaks was sensitive to the endoglycosidase. Instead, other indigestible material was separated from the digested glycopeptides after treatment with endo-β-*N*-acetylglucosaminidase D. This material appeared visible already after digestion with neuraminidase, and is supposed to be due to incomplete removal of sialic acid. This material amounted to 50%, 27% and 33% of GPI, GP IIA and GP IIB respectively. The insensitivity of certain sialic acid residues on Thy-1 to neuraminidase has previously been shown (Carlsson & Stigbrand, 1982). The nature of these residues remains to be elucidated. As shown in Figs. 5(*m*)–5(*o*), the endoglycosidase-digested material was not homogeneous but each glycopeptide consisted of at least two components. These heterogeneities were shown to be due to differences in the peptide moieties (see below).

As discussed above, *N*-linked glycans may contain terminal fucose α-linked to the *N*-acetylglucosamine bound to asparagine of the peptide. In order to investigate this for the Thy-1 glycopeptides, digestions were performed with α-L-fucosidase on the endoglycosidase-digested material. The results are shown in Figs. 5(*p*)–5(*r*). As predicted from the lentil lectin chromatographies, GP IIB contained fucose whereas GP IIA did not. Also, the size of the major material of GP I decreased after α-L-fucosidase digestion and is therefore considered to contain a fucose residue linked to the innermost *N*-acetylglucosamine. It was, however, not possible to decide whether the endoglycosidase-insensitive material of GP I also contained fucose, since removal of one fucose residue in that molecular-mass region did not significantly alter the migration on the gel (results not shown).

Gel filtration of Thy-1 GP III is shown in Fig. 6(*a*). The elution pattern revealed at least two components with different molecular masses. When this material was digested with endo-β-*N*-acetylglucosaminidase D (Fig. 6*b*), approximately half of the glycopeptides were shifted to lower-M_r species, indicating that these structures possess a terminal α-(1→3)-linked mannose residue. Further digestion was carried out with endo-β-*N*-acetylglucosaminidase H (Fig. 6*c*), which cleaves 'high-mannose' structures between the core *N*-acetylglucosamine residues, independently of whether the α-(1→3)-linked mannose is terminal or not (Tai *et al.*, 1977*b*). By digestion with this enzyme, all of Thy-1 GP III was converted into smaller glycopep-

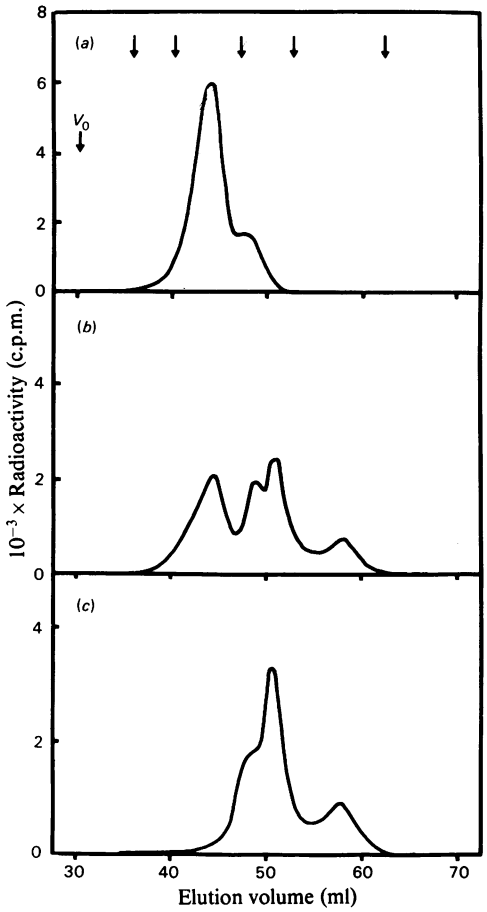


Fig. 6. *Gel filtration of intact and glycosidase-treated Thy-1 glycopeptide III*
Conditions were the same as in Fig. 5. (*a*) Untreated; (*b*) treated with endo-β-*N*-acetylglucosaminidase D; (*c*) treated with endo-β-*N*-acetylglucosaminidase H.

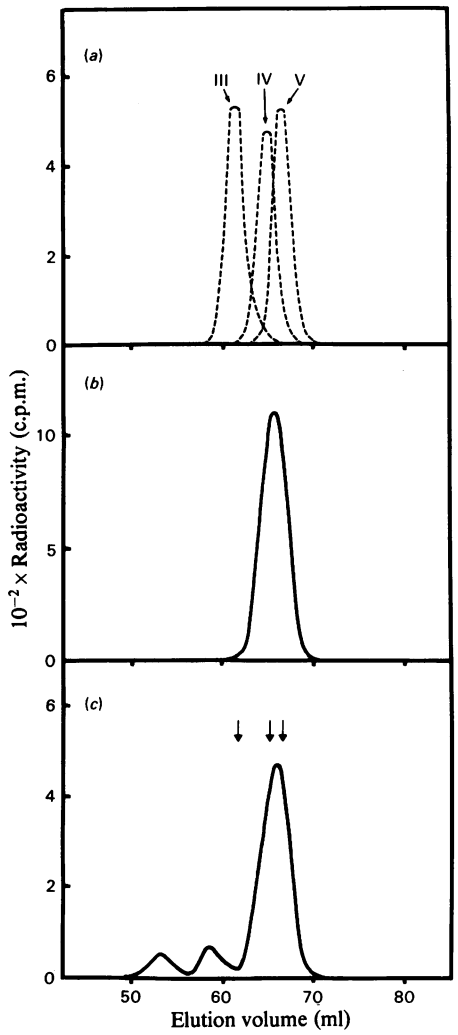


Fig. 7. *Gel filtration of oligosaccharide alcohols*
Glycopeptides were digested with endo-β-*N*-acetylglucosaminidase H, and the released oligosaccharides were reduced with NaB^3H_4. Gel filtration was performed under the same conditions as in Figs. 5 and 6. (*a*) Oligosaccharide alcohols from ovalbumin GP III, GP IV and GP V. (*b*) Mixture (1 : 1) of oligosaccharide alcohols from ovalbumin GP IV and GP V. (*c*) Oligosaccharide alcohols from Thy-1 GP III. Arrows denote the elution of the oligosaccharides shown in (*a*).

tides, confirming that this structure is of 'high-mannose' type, as suggested from concanavalin A experiments. The heterogeneity still seen after endoglycosidase digestions is probably due to peptides of different size. Since the major peak of digested Thy-1 GP III was eluted as having a larger molecular mass than that of digested GP I, GP IIA and GP IIB [compare Figs. 5(*p*)–5(*r*) and 6(*c*)], it follows that GP III contains a longer peptide chain attached to the carbohydrate.

In order to make a more direct comparison betwen the 'high-mannose' structures of Thy-1 and the glycans of the marker glycopeptides, independently of the peptide moieties, oligosaccharides were prepared by digestion with endo-β-*N*-glucosaminidase H and converted into sugar alcohols by reduction with NaB^3H_4. Fig. 7(*a*) shows the gel filtration of oligosaccharide alcohols from ovalbumin GP III, IV and V. A difference of one mannose residue is clearly detected. Oligosaccharide alcohols from Thy-1 GP III are shown in Fig. 7(*c*). One major broad peak, eluted at the positions of ovalbumin GP IV and V oligosaccharides, was obtained, together with two small peaks of larger molecular mass. When a mixture of oligosacchar-

ides from ovalbumin GP IV and V was run on the gel-filtration column (Fig. 7*b*), the elution position and peak shape were almost identical with those of Thy-1 GP III oligosaccharide. It is therefore suggested that the major part of the Thy-1 'high-mannose' chains is a mixture (approx. 1:1) of structures represented by ovalbumin GP IV and V. This suggestion is also consistent with the result shown in Fig. 6. The small peaks of larger molecular mass in Fig. 7(*c*) may represent precursor oligosaccharide structures that were under processing by the cell. Such structures are predicted to occur since Thy-1 was purified from whole-cell microsomal membranes rather than from pure plasma membranes (Carlsson & Stigbrand, 1983*b*).

Glycan distribution on the peptide backbone

It is known that, even after extensive Pronase digestions of glycoproteins, not only asparagine but also a few additional amino acids often remain in the glycopeptide fraction (Takasaki *et al.*, 1982). One of the few exceptions is ovalbumin GP which can be obtained containing only one amino acid (i.e. asparagine) attached to carbohydrate (Huang *et al.*, 1970). However, information on the additional amino acids can be used to locate the isolated sugar structures on the peptide backbone, if the amino acid sequence is known. The amino acid sequence of mouse Thy-1 was recently reported by Williams & Gagnon (1982), and in Fig. 8 the sequences at the glycosylation sites are shown. Table 1 shows the amino acid compositions of unfractionated and isolated Thy-1 glycopeptides. Since the glycopeptide mixture contained threonine but no phenylalanine or leucine, the threonine must be located at position no. 25. It follows that the glycopeptide mixture contained four asparagine residues per molecule of Thy-1, and the amount of the other amino acids was calculated in relation to that number. Approximately one residue each of glutamic acid, alanine and serine was present per molecule of Thy-1. These amino acids represent residues linked to the amino groups of the glycosylated asparagine residues, and were obviously resistant to Pronase treatment. Lysine could originate from either position 26 or position 100. The presence of glycine was assumed to be due to contamination, since the sequences near the glycosylation sites were lacking that amino acid.

Table 1. *Amino acid composition of isolated Thy-1 glycopeptides*

	Amino acid composition				
	(mol of amino acid/	(mol of amino acid/mol of glycopeptide)			
Detectable amino acids	mol of Thy-1) Unfractionated	GP I	GP IIA	GP IIB	GP III
Asx	4.00	1.00	1.00	1.00	2.00
Thr	0.87	<0.10	<0.10	<0.10	0.64
Ser	1.03	0.31	0.92	0.48	0.17
Glx	0.90	0.13	0.23	0.17	0.73
Gly	0.17	0.13	0.23	0.18	0.17
Ala	0.75	0.60	0.13	0.50	0.10
Lys	1.13	0.16	0.46	0.29	0.53

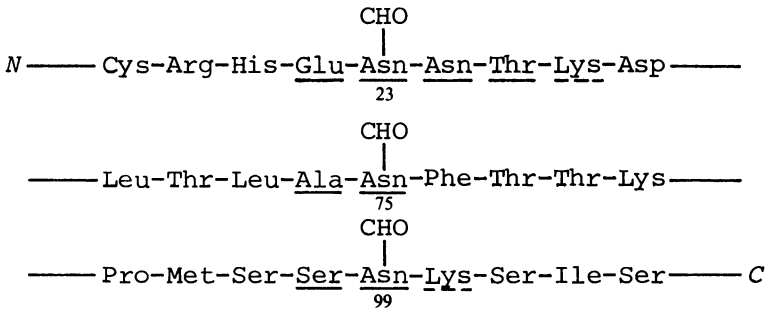


Fig. 8. *Amino acid sequences of mouse Thy-1 at the glycosylation sites*
Amino acids of the isolated glycopeptides are underlined. The broken lines indicate partially present amino acids. The carbohydrate moieties are indicated by CHO. The amino acid sequence was taken from Williams & Gagnon (1982).

Table 2. *Distribution of the oligosaccharide structures*
The values listed for the different glycosylation sites represent the distribution (%) of each oligosaccharide structure and are based on amino acid composition of the glycopeptides. The total relative amount (%) of the oligosaccharides was based on radioactivity measurements.

Oligosaccharide	Distribution at glycosylation sites (%) Asn-23	Asn-75	Asn-99	Total relative amount (%)
I	12	58	30	27
IIA	18	10	72	22
IIB	15	43	42	20
III	73	10	17	31
				100

Since glutamic acid, alanine and serine could be used as marker amino acids for the three glycosylation sites respectively, it could be shown that the isolated glycan structures were distributed in a mixed fashion on the peptide (Table 1). In the case of the 'high-mannose' chain, however, most of this structure was located at position Asn-23, as concluded from the high glutamic acid value and the low values of alanine and serine. Since this glycopeptide partially also contained lysine, the largest peptide moiety contained as many as five amino acid residues. This finding agrees well with the large molecular mass obtained after endoglycosidase digestion of GP III (see Fig. 6*c*). The major part of the other glycan structures was distributed between positions Asn-75 and Asn-99. The peptide moieties of the isolated glycopeptides are underlined in Fig. 8. The lysine residue at position 100 was shown to be partially present.

Table 2 shows the distribution of each of the four oligosaccharide structures at the three glycosylation sites. The values shown are percentage values of each 'marker' amino acid (i.e. glutamic acid, alanine and serine) in relation to the sum of these amino acids for each structure. Also shown in Table 2 is the relative amount of each oligosaccharide structure, determined on the basis of radioactivity measurements. It can be concluded that most of GP I is located at position Asn-75, GP IIA at Asn-99 and GP III at Asn-23 and that GP IIB is equally distributed between positions Asn-75 and Asn-99.

Discussion

Mouse brain Thy-1 was previously shown to possess three sites to which carbohydrate was attached, through *N*-linkage to asparagine (Williams & Gagnon, 1982). All *N*-linked glycans in Nature seem to have a common core with the structure Man-α-(1→3)-[Man-α-(1→6)-]Man-β-(1→4)-GlcNAc-β-(1→4)-GlcNAc-(1→)Asn. To this core further mannose ('high-mannose chains') or *N*-acetylglucosamine, galactose, fucose and sialic acid ('complex-type chains') can be added. Also a 'hybrid-type' of these structures can occur. Since mouse thymocyte Thy-1 was shown to lack *N*-acetylgalactosamine (Carlsson & Stigbrand, 1983*b*) (the sugar that constitutes the linkage of *O*-linked glycans to the peptide) and since no positions other than at asparagine residues 23, 75, and 99 fulfilled the sequence requirement for *N*-linkage of carbohydrate (i.e. -Asn-Xaa-Thr/Ser-) (Struck & Lennartz, 1980), only three *N*-linked chains per molecule were to be expected. This was confirmed in our study, since no other amino acids, with the exception of glycine (see above), than those belonging to the above-mentioned sites were isolated together with the carbohydrate.

From lectin-binding properties, sensitivity to exo- and endo-glycosidases and estimations of M_r values by gel filtration, taking into account what is generally known about *N*-linked oligosaccharides, the structures shown in Fig. 9 are proposed for thymocyte Thy-1 glycans. Since no information about the linkage positions between the monosaccharides was obtained in the present study, these are omitted, even though some general rules seem to exist (Sharon & Lis, 1982). Some alternatives to the structures shown are possible. (1) The sialic acid residues may be linked differently. Several structures have been shown to contain sialic acid linked to *N*-acetylglucosamine of the branch (Mizuochi *et al.*, 1979), and the sialic acid residues may also occur linked to each other (Finne, 1982). The linkage shown is, however, the most commonly found in the literature. (2) Analysis of the glycan structures of the whole glycopeptide fraction of calf thymocyte membranes (Kornfeld, 1978) has revealed the presence of several galactose residues on the same branch. This arrangement is also possible for mouse thymocyte Thy-1 glycans, although less likely since GP I and GP IIA behaved very similar to fetuin GP and transferrin GP respectively after removal of sialic acid. (3) A 'bisecting' *N*-acetylglucosamine residue linked to the β-linked mannose of the core may be present. This possibility has to be considered, especially since some of the Thy-1 molecules (approx. 25%) bind to wheat-germ agglutinin (Carlsson & Stigbrand, 1982). This lectin has been shown to have affinity for such structures (Yamamoto *et al.*, 1981). If present, the additional *N*-acetylglucosamine residues can reside only in GP I and/or GP III, since a biantennary structure of that type has been shown not to bind to concanavalin A (Baenziger & Fiete, 1979*b*). (4) The structures of

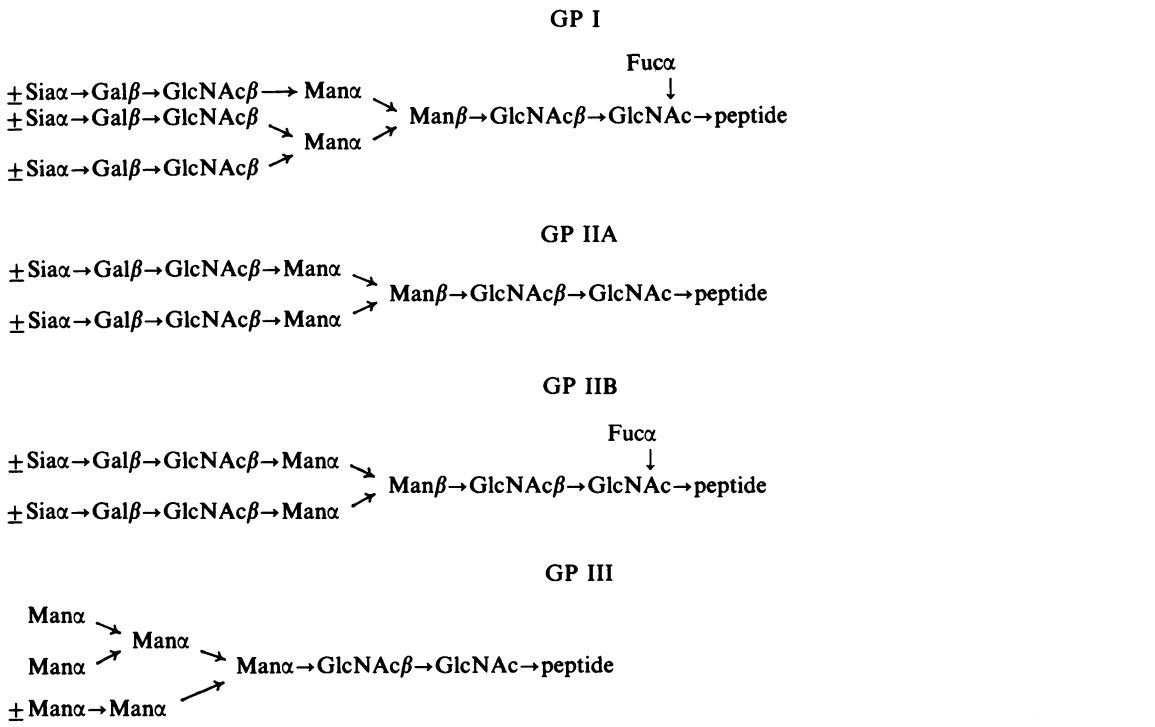


Fig. 9. *Proposed structures of thymocyte Thy-1 oligosaccharides*

the 'undigested' glycopeptides remaining in the large-molecular-mass region after endoglycosidase treatment (see Fig. 5) may be different from those shown in Fig. 9. The differences, if any, are, however, suggested to be minor, since the 'undigested' glycopeptides had the same affinity to the lectins and the same molecular mass as the glycopeptides that could be digested.

The structures investigated in the present study have to be confirmed by use of other methods, in order to elucidate the above-mentioned alternatives and to establish the linkage positions between the monosaccharides. Also, the nature of the sialic acid residues has to be determined, since as many as 20 different variants have been found in Nature (Schauer, 1982). This is especially interesting since certain glycan-bound sialic acids have been shown to be resistant to neuraminidase digestion (Schauer, 1982).

The isolated glycan structures were shown to be distributed in a mixed fashion at the three glycosylation sites, i.e. one particular asparagine residue is associated with glycans of different types. In order to estimate the proportion of each glycan structure at the different glycosylation sites, the values for the distribution of the glycans shown in Table 2 were multiplied by the relative amount

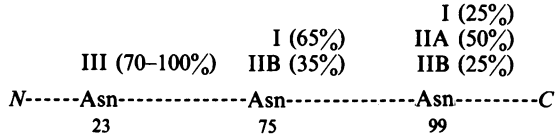


Fig. 10. *Glycosylation of thymocyte Thy-1*
The percentage values show the relative occurrence of the different glycan structures at each glycosylation site.

of each structure. The sum of the proportions of the glycans were 33%, 30% and 38% for the glycosylation sites Asn-23, Asn-75 and Asn-99 respectively, which indicates that all three sites are fully glycosylated. If the sum is put to 100% at each site, the approximate proportion of the major glycan structures will be as shown in Fig. 10. Only those structures that contributed to more than 15% at each site were taken into account in the calculations. At the glycosylation site Asn-23 the predominant structure is of 'high-mannose' type. The other structures may also be present at that site, since the 'complex-type' chains were associated with small but significant amounts of glutamic acid. The most abundant of those structures was GP IIA, which

amounted to 12% of the glycans at that site. However, since the 'complex-type' glycopeptides had undetectable amounts of threonine, whereas GP III and the unfractionated mixture contained almost equal amounts of threonine and glutamic acid (see Table 1), it is possible that the presence of glutamic acid in GP I, GP IIA and GP IIB was due to contamination. Further studies have to confirm whether position Asn-23 is invariably glycosylated by a 'high-mannose' chain or not.

The most abundant structures at position Asn-75 are GP I (65%) and GP IIB (35%). Only very small amounts, if any, are present of the other structures as revealed by their low content of alanine. If all of GP I contains fucose (see above), it follows that all glycans at Asn-75 are fucosylated, and are of either tri- or bi-antennary type. Position Asn-99 was found to be associated with all three types of 'complex' structures, the most abundant being GP IIA (50%).

As mentioned in the Results section, significant amounts of 'high-mannose' chains are to be expected at all glycosylation sites, since a proportion of the Thy-1 molecules purified were under processing inside the cell. The small amounts of alanine and serine in GP III may be a reflection of this phenomenon. It remains, however, to be determined if 'high-mannose' chains can be present at more than one position on cell-surface-bound Thy-1.

It is obvious that the glycans at positions Asn-75 and Asn-99 can be processed in different ways. It is not known, however, whether a certain structure at one position is coexpressed with a certain structure at the other position, or if the different glycans are distributed randomly in the proportions shown in Fig. 10. If the latter is the case, it can be calculated that half of the Thy-1 molecules contain at least one chain of structure IIB and would consequently bind to lentil lectin. This is exactly what was found experimentally (Carlsson & Stigbrand, 1982). The non-binding fraction would contain structures I and IIA at the two sites instead of IIB, which will give that fraction a slightly larger mean molecular mass compared with the lentil-lectin-binding fraction. This is also in accordance with previous results (Barclay *et al.*, 1976). The 'blocking residue' previously discussed would thus be a third antenna on the 'complex-type' oligosaccharides. By analysis with sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and gel filtration, thymocyte Thy-1 was shown to be very heterogeneous, containing several molecular species differing in size (Carlsson & Stigbrand, 1983*b*). On the basis of the results reported in the present paper we suggest that the size-heterogeneity is due to glycans of different structures, present at positions Asn-75 and Asn-99.

From the proposed structures shown in Fig. 9 and the relative distribution shown in Fig. 10, theoretical calculations on the mean number of each monosaccharide residue per Thy-1 molecule can be made. The values obtained were: 1.5 fucose, 11.5 mannose, 4.9 galactose, 10.9 *N*-acetylglucosamine and about 2.5 residues of sialic acid. These values agree with those obtained experimentally, i.e. 1.6 fucose, 9.2 mannose, 6.3 galactose, 9.5 *N*-acetylglucosamine and 3.1 sialic acid residues (Carlsson & Stigbrand, 1983*b*). The theoretical fucose value was calculated assuming that GP I was fully fucosylated. The validity of this assumption is thus strengthened by the experimental value.

It is well known that the thymocyte population is not homogeneous, but can be divided into subfractions that differ by chemical and functional criteria (for a review see Droege & Zucker, 1975). It is also generally accepted that the immature lymphocytes within the thymus have already acquired their antigen-specificities, and it seems therefore reasonable to assume that very few cells are identical within the thymus. In this respect, it is of fundamental importance to determine whether the glycosylation varies between different cells and furthermore how it changes during the differentiation process, in order to elucidate the physiological roles of protein-bound glycans at the lymphocyte surface. Since Thy-1 is a major constituent of the glycoproteins in the thymocyte plasma membrane and possesses a high proportion of carbohydrate, this molecule is a good candidate for carbohydrate-mediated interactions with cells or molecules within the thymus or in the periphery.

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