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N-linked oligosaccharides of the murine transferrin receptor from a plasmacytoma cell line. Comparison with total cellular *N*-glycans

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The N-linked oligosaccharides synthesised by the murine plasmacytoma cell line NS-1 have been analysed by lectin affinity chromatography on columns of immobilised concanavalin A (Con A), *Lens culinaris* (lentil), *Ricinus communis* agglutinin (RCA) and leuko-phytohemagglutinin (L-PHA). The majority of complex *N*-glycans in this transformed cell line were branched structures with only a low level of biantennary complex chains detected. The analysis showed the major complex *N*-glycan fraction consisted of a minimum sialylated triantennary structure. [^{3}H]Mannose-labelled transferrin receptor was isolated from NS-1 cells by immunoprecipitation followed by electroelution from SDS polyacrylamide gels. The isolated receptor was digested with Pronase and the ^{3}H-labelled glycopeptides analysed by lectin affinity chromatography. Analysis by Con A-Sepharose indicated that approx. 50% of the labelled glycopeptides were branched complex *N*-glycans (unbound fraction) while the remainder were oligomannose structures (strongly bound). The presence of tri and/or tetraantennary structures in the Con A unbound fraction was further suggested by the interaction of 61% of the fraction with L-PHA. The lectin profiles obtained for the complex *N*-glycans of the transferrin receptor glycopeptides were similar to those for the total cellular glycopeptides of NS-1 cells. Reverse-phase HPLC analysis of tryptic glycopeptides of the isolated [^{3}H]mannose-labelled transferrin receptor gave three ^{3}H-labelled peaks, indicating that all three potential *N*-glycosylation sites on the receptor are utilised. The Con A-Sepharose profiles of the three fractions indicated the presence of branched complex *N*-glycans and high mannose chains at each site. The profiles of two of the tryptic glycopeptide fractions were very similar, while the third had a higher content of oligomannose oligosaccharides.

Introduction

The transferrin receptor is a major cell surface glycoprotein on all proliferating mammalian cells [1–3]. It has an essential role in the transport of iron into cells and this occurs via the receptor-mediated endocytosis pathway. The human and murine transferrin receptors are disulphide-bonded homodimers with a subunit molecular weight of 90000–95000 [4–6]. The receptor contains N-linked oligosaccharides [4,7,8] and from the amino acid sequence there are three potential *N*-glycosylation sites in the extracellular domains of both the human and murine receptors [9–11]. However, the number of these sites glycosylated is not known. Structural information on the oligosaccharide chains of this receptor is, to date, limited to glycosidase studies which suggest that both high mannose and complex *N*-glycans are present on the mature form of both the human and murine receptor [4,7,8,12]. Recent studies on both the human and murine receptor have suggested that, although the carbohydrate may not be necessary for specific binding to transferrin, the affinity of the interaction with transferrin is influenced by the presence or absence of the carbohydrate [12,13,14].

To gain more insight into the function of the carbohydrate of the transferrin receptor we have investigated the structures of the *N*-glycans of the murine transferrin receptor from a plasmacytoma cell line. We have compared the structures found on the receptor to the total cellular *N*-glycans produced by this cell line to determine if the profile of structures are specific for this glycoprotein receptor or reflect the total population of

Abbreviations: L-PHA, leuko-phytohemagglutinin; RCA, *Ricinus communis* agglutinin.

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N-glycans synthesised by this cell type. In addition, to establish the number of glycosylation sites utilised on the transferrin receptor, we have also isolated the individual tryptic glycopeptides from the transferrin receptor and partially characterised the oligosaccharide structures at each glycosylation site.

Materials and Methods

Cells. NS-1/1. Ag4.1 (NS-1) cells, a murine plasmacytoma cell line from BALB/c mice (15), were maintained in exponential growth in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin and 0.1% (w/v) streptomycin.

Radiolabelling of NS-1 cells. NS-1 cells ($2 \cdot 10^6$ cells, viability > 95%) were washed twice in sterile PBS and resuspended at a density of $8 \cdot 10^5$ cells/ml in low glucose (4 mM) RPMI 1640 supplemented with 10% fetal calf serum and 2 mM glutamine. Cells were labelled for either 5 or 20 h with 10–40 μCi/ml of [2-^{3}H]mannose (Amersham, 19 Ci/mmol) or for 5 h with 5 μCi/ml [5,6-^{3}H]fucose (Amersham, 45 Ci/mmol). Prior to addition to cells the isotopes were dried to remove ethanol, redissolved in low glucose RPMI, and filter sterilised.

Preparation of total cellular glycopeptides. Metabolically labelled cells were washed at least twice with PBS, and the final cell pellets suspended in 0.5 ml of 0.1 M Tris-HCl buffer (pH 8.0) containing 5 mM $CaCl_2$ and 0.02% sodium azide, and then placed in a boiling water bath for 10 min. After cooling to 37°C, 50 μl of 5 mg/ml of Pronase (Calbiochem-Behring Cat. No. 537088), which had been pretreated at 50°C for 30 min to destroy possible interfering enzyme activities, was added and the mixture incubated for 72 h at 37°C. Fresh aliquots (50 μl each) of Pronase were added after 24 and 48 h incubation. After 72 h, the digest was treated at 100°C for 10 min to inactivate the Pronase, and centrifuged at $10000 \times g$ for 10 min. Unincorporated [^{3}H]mannose was separated from the labelled glycopeptides by gel filtration on a column (1.6 × 26 cm) of Bio-Gel P2, 200–400 mesh (Bio-Rad), equilibrated and eluted with water with a flow rate of 16 ml/h. Fractions (2 ml) were collected, aliquots removed for scintillation counting, and the glycopeptide fraction pooled.

Isolation of mannose-labelled transferrin receptor. Cells metabolically labelled for 20 h were washed twice with PBS, pelleted at $500 \times g$ for 5 min at room temperature, and extracted in 0.5 ml of PBS containing 0.5% Nonidet P-40 at 0°C for 30 min. Nuclei and cell debris were removed by centrifugation at $1000 \times g$ for 15 min at 4°C and the [^{3}H]mannose-labelled extracts further centrifuged at $89000 \times g$ in a Beckman TLA 100.2 rotor for 1 h at 4°C. The transferrin receptor was immunoprecipitated as previously described [8,12] using rat monoclonal anti-(mouse transferrin receptor) antibodies (class IgM) followed by the second antibody mouse anti-(rat κ chain) antibody and *Staphylococcus aureus* cells.

Preparative SDS polyacrylamide gel electrophoresis. Electrophoresis of the immunoprecipitates was carried out under reducing conditions on 7.5% SDS/polyacrylamide gels using the buffer system of Laemmli [16]. Molecular weight standards (Bio-Rad) were run at each side of the sample. Gels were stained in 0.2% Coomassie blue R-250 for 5 min and destained in 12% ethanol/7% acetic acid for 30 min. The labelled receptor was then recovered from the gel by removing a gel slice spanning the 95000 dalton molecular mass marker region and soaking the gel slice in water for 30 min followed by a 30 min incubation in Tris-glycine buffer (pH 8.3) containing 50 mM dithiothreitol to ensure that the sample was completely reduced. The acrylamide gel slice was then loaded into a glass elution tube, and the labelled receptor eluted electrophoretically at 50 V for 2 days as described by Stearne et al. [17].

Reduction and carboxymethylation. The electrophoretically eluted receptor sample (in SDS) was made 10 mM in dithiothreitol and heated to 100°C for 5 min. After cooling, recrystallised iodoacetic acid was added to a final concentration of 50 mM, and the sample held in the dark at 37°C for 45 min before precipitation with methanol.

Methanol precipitation and digestion of isolated ^{3}H-labelled receptor. The reduced and carboxymethylated sample was transferred into a siliconised Corex tube, 100 μg of carrier bovine serum albumin was added, then 9 vol. of methanol (precooled to −20°C) was added and the mixture held at −20°C overnight to precipitate the protein. Preciptitates were recovered by centrifugation for 45 min at $16000 \times g$ at −5°C in a Sorvall HB-4 rotor as described by Stearne et al. [17]. Samples were either digested with Pronase as described above or with trypsin as follows. The sediment was resuspended in 100 μl of 10 mM Tris-HCl buffer (pH 8.0) containing 2 mM $CaCl_2$, 20 μg trypsin (Worthington) was added and the mixture incubated at 37°C for 48 h and the sample then fractionated on reverse-phase HPLC.

Reverse-phase HPLC fractionation of tryptic glycopeptides. The tryptic glycopeptides were separated by reversed-phase HPLC on a Bondapak C_{18} column (Waters) using a flow rate of 1 ml/min and a linear gradient of 0 to 50% solvent B, where the starting solvent A was H_2O/0.1% trifluoroacetic acid (TFA) and solvent B was acetonitrile/0.1% TFA. Fractions (0.5 ml) were immediately neutralised with 1 M Tris buffer (pH 8.4) and aliquots removed for scintillation counting. Recovery of radioactivity from the column ranged over 40–70%.

Pooled fractions were desalted on a Bio-Gel P2 column and further digested with Pronase as described above.

Desialylation of glycopeptides. Samples were desialylated by mild acid hydrolysis. Glycopeptide samples were dried on a rotary evaporator and heated at 100°C for 5 min in 2 M acetic acid. Hydrolyzed samples were purified immediately by gel filtration on Bio-Gel P2.

Lectin affinity chromatography. Glycopeptides were fractionated by lectin affinity chromatography essentially as described by Cummings and Kornfeld [18] and Narasimhan et al. [19]. All columns were run at room temperature.

Con A-Sepharose 4B. Glycopeptide fractions were applied to a column (0.7 × 8 cm) of Con A-Sepharose (Pharmacia) equilibrated in 10 mM Tris-HCl (pH 7.5) containing 0.1 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 0.02% NaN_3. Chromatography was performed with a flow rate of approx. 15 ml/h. The column was washed with 25 ml of the starting buffer followed by 30–40 ml of the buffer containing 10 mM methyl α-D-glucoside followed by 35 ml of the buffer containing 500 mM methyl α-D mannoside. 1 ml fractions were collected and 500 μl aliquots were removed for liquid scintillation counting.

Lentil lectin-Sepharose 4B. Chromatography on a lentil lectin-Sepharose 4B (Pharmacia) column (0.7 × 7 cm) was performed as for Con A-Sepharose, with the exception that the column was washed first with 25 ml of the buffer, followed by 25 ml of buffer containing 200 mM methyl α-D-mannoside.

L-PHA-Agarose. Glycopeptides were applied to a column (0.7 × 10 cm) of L-PHA-Agarose (E-Y Laboratories, San Mateo, CA Lot No. 031115) equilibrated in PBS containing 0.02% NaN_3. The column was run in PBS with a flow rate of 5 ml/h and 0.5 ml fractions were collected.

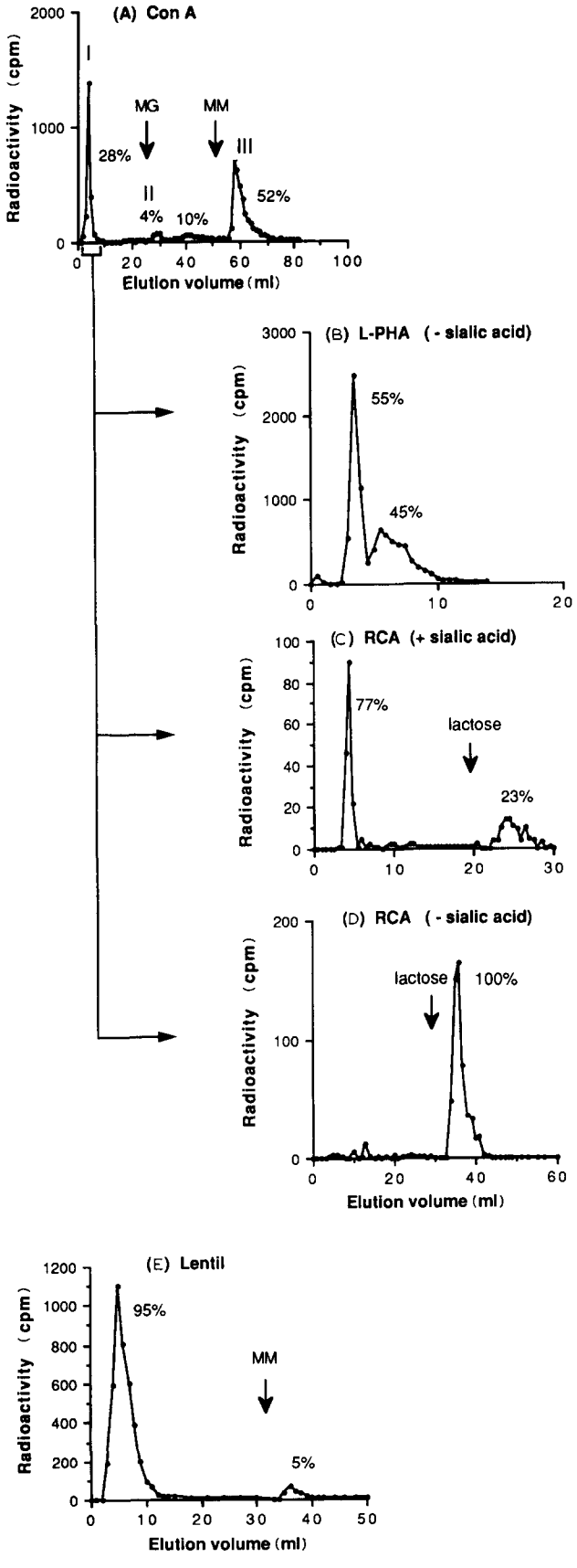
RCA-Agarose. RCA-Agarose was purchased as RCA-1 (RCA-120)-Agarose at 6–10 mg/ml (E-Y Laboratories, San Mateo, CA; Lot No. 041015). Glycopeptide fractions were applied to a column (0.7 × 20 cm) of RCA-Agarose equilibrated in 10 mM Tris-HCl (pH 7.5) containing 0.1 M NaCl and 0.02% NaN_3. Chromatography was performed with a flow rate of 6 ml/h. The column was washed with 20–40 ml of starting buffer followed by 15 ml of buffer containing 0.1 M lactose, and 0.5 ml fractions were collected.

Results

Fractionation of glycopeptides of murine plasmacytoma NS-1 cells by sequential lectin chromatography

The N-linked oligosaccharide structures synthesised by NS-1 cells were initially characterised so that a comparison could be made with the structures found on the transferrin receptor. Total cellular glycopeptides were obtained by Pronase digestion of cell glycoproteins metabolically labelled with [^{3}H]mannose and these labelled glycopeptides were characterised by serial lectin affinity chromatography. Firstly, the labelled glycopeptides were fractionated by chromatography on Con A-Sepharose (Fig. 1A). Previous studies by a number of workers [20,21] have shown that tri- and tetraantennary glycopeptides (fraction I) pass directly through Con A-Sepharose while biantennary glycopeptides (fraction II) interact with the column and can be eluted with 10 mM methyl α-D-glucoside. On the other hand hybrid and oligomannose glycopeptides (fraction III) interact strongly with Con A and should elute with 500 mM methyl α-D-mannoside. Con A-Sepharose chromatography of [^{3}H]mannose-labelled glycopeptides from NS-1 cells resulted in two major fractions; an unbound fraction and a fraction eluting with 500 mM methyl α-D-mannoside (Fig. 1A). The unbound fraction presumably represents branched complex *N*-glycans. Very little material (4%) eluted in the presence of 10 mM methyl α-D-glucoside (fraction II), indicating a very low content of biantennary complex *N*-glycans in NS-1 cells. The material that eluted as a very broad retarded peak with 10 mM methly α-D-mannoside probably represents fraction III glycopeptides which have started to elute from the Con A-Sepharose column with 10 mM sugar; variation in the elution of fraction C glycopeptides on different batches of Con A-Sepharose has previously been reported [21]. This conclusion is also supported by the earlier finding that the glycopeptides from either swainsonine- or castanospermine-treated NS-1 cells showed a considerable increase in this material eluting as a broad retarded peak on this batch of Con A-Sepharose 4B [12]. Con A fraction III (Fig. 1A) probably represents predominantly high mannose oligosaccharides, as hybrid structures could not be detected in this fraction. The absence of hybrid structures is based on the following observations. Firstly, this fraction did not label significantly with [^{3}H]fucose (Fig. 2A), and hybrid structures often have a core fucose. Secondly, the Con A fraction III material, even after treatment with mild acid, passed directly through an RCA column which indicates an absence of either terminal or sub-terminal galactose residues (results not shown).

The total labelled glycopeptides were also analysed on lentil lectin-Sepharose. The majority of the [^{3}H]mannose-labelled glycopeptides passed directly through a column of lentil lectin-Sepharose (Fig. 1E). This indicates that there is only a low content of core fucosylated biantenary complex structures or core fucosylated triantennary structures with an α-mannose residue substituted at positions C-2 and C-6 by GlcNAc (triantennary type II structures). Fucosylated glycopeptides were directly examined by the analysis of [^{3}H]fucose-labelled glycopeptides from NS-1 cells on Con A-Sepharose



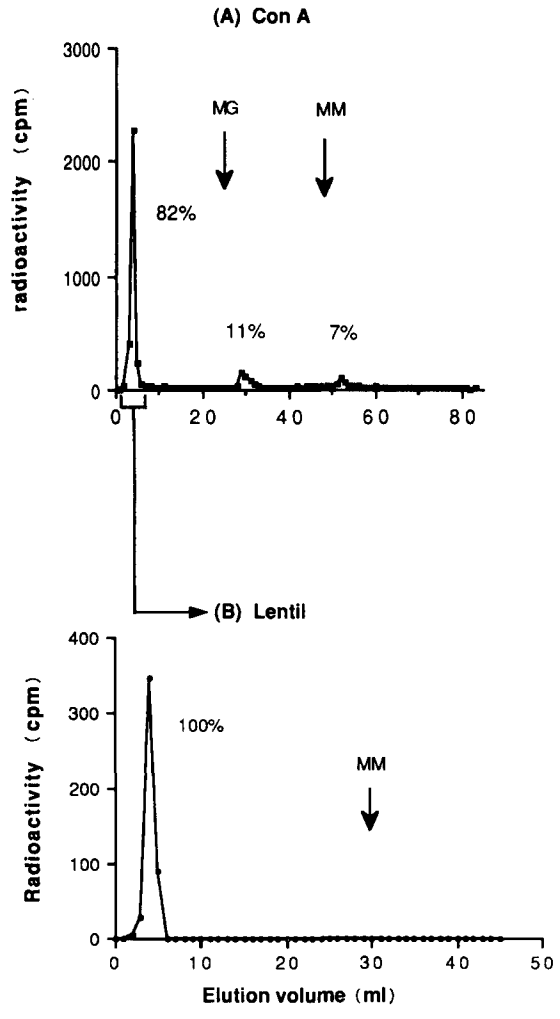


Fig. 2. Analysis of [^{3}H]fucose-labelled NS-1 glycopeptides. NS-1 cells were labelled with [^{3}H]fucose and the [^{3}H]glycopeptides obtained by Pronase digestion of cell pellets. An aliquot (containing 19000 cpm) was chromatographed on Con A-Sepharose (A) and the Con A fraction I recovered and analysed further on lentil lectin-Sepharose (B) as indicated in Fig. 1. Recoveries of radioactivity from the columns were 86 and 100%, respectively.

Fig. 1. Serial lectin chromatography of [^{3}H]mannose-labelled glycopeptides of NS-1 cells. NS-1 cells were labelled with [^{3}H]mannose and the [^{3}H]glycopeptides obtained by Pronase digestion of the cell pellets. (A) Chromatography of an aliquot (containing 20500 cpm) of the [^{3}H]glycopeptides on a Con A-Sepharose column. The arrows indicate the start of elution with 10 mM methyl α-D-glucoside (MG) and 500 mM methyl α-D-mannoside (MM), respectively. 1 ml fractions were collected and 500 μl aliquots counted for radioactivity. Recovery of radioactivity from the column was 80%. The Con A unbound fraction (fraction I) was then applied to a column of RCA-Agarose (D), with a recovery of 70%. The Con A fraction I was also desialylated as described in Materials and Methods and applied to columns of L-PHA-Agarose (B) RCA-Agarose (D), with recoveries of 82 and 75%, respectively. Material that bound to the RCA columns was eluted with 0.1 M lactose. The total cellular [^{3}H]glycopeptides were also chromatographed on a column of lentil lectin-Sepharose (E). The arrow indicates the start of elution with 200 mM methyl α-D-mannoside. Recovery from the column was 97%.

(Fig. 2A). The majority (82%) of the [^{3}H]fucose-labelled glycopeptides passed directly through the column, and 11% bound weakly and eluted sharply with 10 mM methly α-D-glucoside. This latter fraction probably represents a small proportion of fucosylated biantennary glycopeptides. The [^{3}H]fucose labelled glycopeptides that passed through the Con A column also passed directly through a lentil lectin-Sepharose column (Fig. 2B), which again indicates an absence of a core fucosylated triantennary type II structures This fraction, therefore, probably represents fucosylated triantennary type I structures (α-linked mannose residue substituted at positions C-2 and C-4 by GlcNAc) and/or fucosylated tetraantennary structures (as well as large fucosylated *O*-glycans).

To determine the content of tri- and tetraantennary complex structures, the [^{3}H]mannose-labelled Con A unbound fraction was analysed on a column of L-PHA Sepharose. L-PHA interacts with tri-and tetraantennary glycopeptides containing outer galactose residues and an α-linked core mannose residue substituted at C-2 and C-6 by GlcNAc residues [22]; the interacting glycopeptides are retarded on a column of L-PHA. Triantennary glycopeptides carrying an α-linked mannose residue substituted at C-2 and C-4 (triantennary type I structures) do not interact with L-PHA [22]. After removing sialic acid 45% of the Con A unbound fraction was retarded on this column (Fig. 1B). This retarded fraction probably represents mainly tetraantennary structures as the trianntennary isomer that interacts with L-PHA (triantennary type II structure) is unusual and, furthermore, as discussed above is absent as the fucosylated species in these cells The presence of α-2,6-linked sialic acid has been reported to influence the interaction of glycopeptides with L-PHA [23,24]. Analysis of the sialylated Con A unbound sample (fraction I) showed that only 13% of the sample was retarded on the column compared to 45% for the desialylated sample, suggesting the glycopeptides are extensivley sialylated (results not shown).

The extent of sialylation of this Con A unbound fraction was assessed by interaction with a column of RCA-Agarose (Fig. 1C and D). The majority (77%) of the sialylated sample passed directly through the RCA column, with the remaining 23% bound strongly to the column and eluted with 0.1 M lactose (Fig. 1C). Treatment of the sample with mild acid to remove terminal sialic acid residues resulted in the majority of the sample binding strongly to the RCA column and eluting at a position consistent with the expected behaviour of a tri- or tetraantennary structures [19] (Fig. 1D). Therefore, the galactose residues of these branched complex *N*-glycans appear to be extensively sialylated.

Overall, these findings imply that the major complex *N*-glycan fraction of NS-1 cells has a minimum of a triantennary structure which is extensively sialylated.

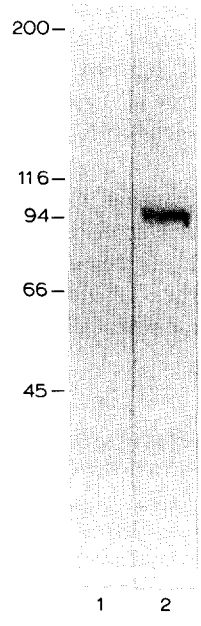


Fig. 3. Immunoprecipitation of [^{3}H]mannose-labelled transferrin receptor from NS-1 cells. NS-1 cells were labelled with [^{3}H]mannose, solubilised and the transferrin receptor immunoprecipitated as described in the Materials and Methods. The immunoprecipitates were separated in a reducing SDS/7.5% polyacrylamide gel and the resulting fluorograph shown. Lane 1, non-specific binding with mouse anti-(rat κ chain) antibody (pre-clear). Lane 2, specific immunoprecipitation of the transferrin receptor. Molecular mass standards are shown (kDa).

Isolation of [^{3}H]mannose-labelled murine transferrin receptor

Transferrin receptors were isolated by immunoprecipitation of detergent extracts of NS-1 cells metabolically labelled with [2-^{3}H]mannose for 21 h. As previous studies have shown that the newly synthesised receptor in NS-1 cells is rapidly converted from an endo H sensitive to a endo H resistant species [8,12], the majority of receptor molecules after a 21 h labelling would be expected to carry fully processed oligosaccharide chains. Approx. 2.5% of the total incorporated mannose was associated with the immunoprecipitated transferrin receptor. Analysis of the immunoprecipitate by SDS-PAGE and fluorography showed a single component of 95 000 daltons under reducing conditions (Fig. 3). This is the expected size of the reduced receptor sub-unit [8,12]. To ensure purity of the [^{3}H]mannose-labelled receptor, the immunoprecipitates were separated by SDS-PAGE and the transferrin receptor recovered from the gel slice by electrophoretic elution and methanol precipitation.

Fractionation of glycopeptides of transferrin receptor by lectin chromatography

The purified [^{3}H]mannose-labelled transferrin receptor was extensively digested with Pronase and the result-

ing glycopeptides analysed by Con A-Sepharose chromatography (Fig. 4). Approx. 49% of the radioactivity was unbound and the remaining 51% bound tightly to the column and was eluted with 500 mM methyl α-D-

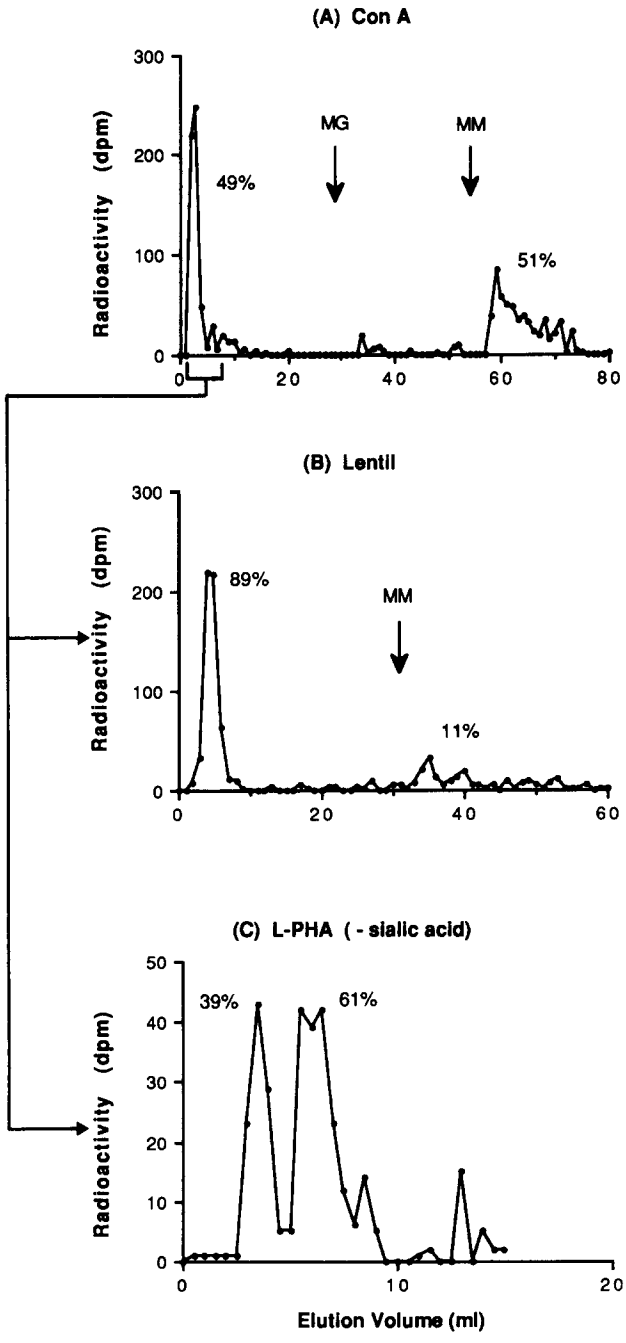


Fig. 4. Analysis of [^{3}H]mannose-labelled transferrin receptor glycopeptides by lectin chromatography. [^{3}H]Mannose-labelled transferrin receptor was isolated by immunoprecipitation from NS-1 cell extracts followed by electroelution from an SDS/polyacrylamide gel. The ^{3}H-labelled glycopeptides, obtained by Pronase digestion of the isolated receptor, were fractionated on a Con A-Sepharose column as indicated in Fig. 1, with a recovery of radioactivity from the column of 78%. The Con A unbound fraction was then either applied to (B) a lentil lectin column (recovery 97%) or (C) desialylated and applied to a L-PHA column (recovery 82%).

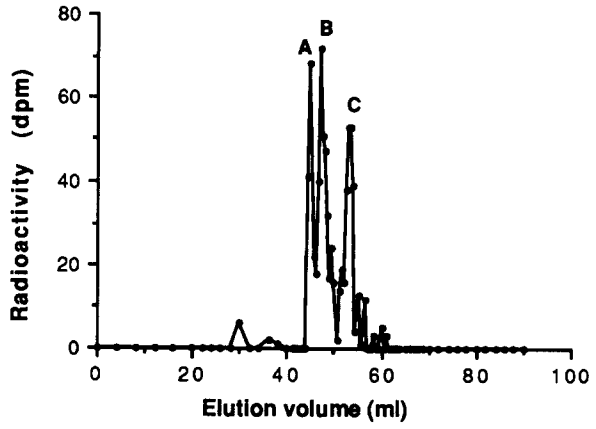


Fig. 5. Reverse-phase HPLC fractionation of [^{3}H]mannose-labelled transferrin receptor tryptic glycopeptides. Tryptic ^{3}H-labelled glycopeptides of the transferrin receptor from a 21 h labelling were fractionated by reverse-phase HPLC on a C_{18} column as described in the Materials and Methods.

mannoside. Therefore, both high mannose and complex glycopeptides are present on the receptor molecule. The low content of weakly bound glycopeptides (Con A fraction II) to the column indicates that biantennary complex structures are only a minor species in the receptor. Approx. 11% of the Con A unbound fraction bound to lentil lectin, indicating the presence of a small amout of core fucosylated triantennary II structures. In addition, about 60% of the desialylated Con A unbound fraction was retarded on L-PHA, indicating the presence of triantennary type II and/or tetraantennary structures. The overall pattern of complex *N*-glycan structures on the receptor is similar to the total population of N-linked oligosaccharides in NS-1 cells.

Isolation and characterisation of [^{3}H]mannose-labelled tryptic peptides from transferrin receptor

Purified ^{3}H-labelled transferrin receptor was digested with trypsin and the resulting tryptic peptides fractionated by reverse-phase HPLC. Three ^{3}H-labelled

TABLE I

Analysis of the three tryptic ^{3}H-labelled glycopeptides of the transferrin receptor by Con A-Sepharose chromatography

The [^{3}H]mannose-labelled tryptic glycopeptides were separated by HPLC, digested with Pronase and analysed by Con A-Sepharose chromatography as described in Materials and Methods. Fraction I is the Con A unbound fraction, fraction II was eluted with 10 mM methyl α-D-glucoside and fractions III eluted with 200 mM methyl α-D-mannoside.

[^{3}H]-Labelled tryptic glycopeptide fraction	Con A-Sepharose fractions (% of recovered radioactivity)		
	I	II	III
A	25	–	75
B	75	–	25
C	65	–	35

peaks were obtained (Fig. 5). As a wide variation in oligosaccharide structure has been reported to have little influence on the retention time of glycopeptides on this HPLC column [25,26], the three ^{3}H-labelled glycopeptides are likely to be derived from three distinct glycosylation sites. The isolated tryptic glycopeptides were then digested with Pronase and analysed by Con A-Sepharose chromatography. The Con A profiles of each glycopeptide fraction showed the presence of both Con A fraction I (tri- and tetraantennary structures) and fraction III (high mannose) in all three samples (Table I). As expected from the earlier analysis there is an absence of fraction II (biantennary structure) in all of the glycopeptide samples. The relative proportions of Con A unbound and Con A strongly bound were different in each glycopeptide sample with glycopeptide A having a higher content of high mannose structures than glycopeptides B and C. These latter two glycopeptides had a ratio of Con A unbound to Con A bound of approx. 3 : 1, whereas glycopeptide A has a ratio of 1 : 3.

Discussion

The murine and human transferrin receptors are disulphide-bonded homodimers with three potential *N*-glycosylation sites on the large carboxy terminal extracellular domain [9–11]. The aim of this study was to analyse the *N*-glycan structures on this receptor and to compare the profile of structures found on the receptor with the total cellular population produced by NS-1 cells. Another objective was to establish how many of the three potential glycosylation sites were utilised in the murine receptor and to determine whether there were differences in the oligosaccharide structures at each glycosylation site. This is the first report investigating the structures of the *N*-glycans of the transferrin receptor and here we have shown that that all three glycosylation sites are utilised in the murine receptor, and that both complex and high mannose N-linked oligosaccharides are found at each-site.

In view of the difficulty of obtaining sufficient quantities of purified transferrin receptor for direct structural analysis of the oligosaccharide chains, we have used the indirect approach of serial lectin chromatography developed by Cumming and Kornfeld [18] which is particularly suited to the analysis of membrane glycoproteins. Fractionation of [^{3}H]mannose labelled glycopeptides on a series of immobilised lectins allowed us to deduce the minimal structures for the major fractions. These structures are based on the known lectin specificities for labelled glycopeptides. Confirmation of these structures will require preparation of a large amount of this membrane receptor for direct analysis.

Analysis of the labelled receptor from cells metabolically labelled with mannose for 21 h showed the presence of both high mannose and complex *N*-glycans. Although biosynthetic intermediates may contribute to the proportion of the high mannose structures detected, it is likely that the majority are derived from the mature form of the receptor as the cells were labelled for 21 h and the half-life of the receptor in this cell line is approx. 60 h [12]. In addition, these results confirm our earlier findings that the transferrin receptor at the cell surface of NS-1 cells is partially sensitive to endo H, indicating the presence of high mannose on the mature receptor [12]. Similar results, based on endo H digestion studies, have also been reported for of the human receptor [4,7].

The majority of complex *N*-glycan structures found in NS-1 cells and on the transferrin receptor from these cells were highly branched structures, namely sialylated triantennary and/or tetraantennary structures. More detailed structural information on the degree of branching of these oligosaccharides could not be obtained with this approach as it is limited by the availability of lectins which can discriminate between branched complex structures. The predominance of highly branched structures in NS-1 cells is of particular interest as this plasmacytoma is a fully transformed cell line. An elevated expression of highly branched complex *N*-glycans has been reported for a number of transformed cells [27,28] and is thought to be due to increased levels of the relevant biosynthetic branching enzymes [29]. The presence of highly branched complex structures on the transferrin receptor no doubt reflects exposure to the biosynthetic glycosylation enzymes of this cell. However, the ability of a particular glycosytransferase to act at a particular site on a glycoprotein can be influenced by the polypeptide backbone [30]. The oligosaccharide intermediates on the transferrin receptor, therefore, must provide the appropriate oligosaccharide acceptor conformations required by the various branching glycosyltransferases.

Each of the three glycosylation sites contained a mixture of high mannose and complex structures. However, one site showed a lower proportion of high mannose oligosaccharides compared to the other two sites. This suggests there may be site-specific glycosylation processing of the transferrin receptor. However, due to shortage of material as well as the limitations of the methods employed, it was not ascertained whether the complex structures differed at each site. A number of studies of multiply glycosylated glycoproteins have shown distinct sets of carbohydrate structures at each individual glycosylation site, for example the murine MHC antigens [26], human tissue plasminogen activator [31] and Thy-1 [32]. This site specific processing of the *N*-glycan chains is thought to be influenced by the local polypeptide environment [30]. The results presented here also indicate there is site-specific processing of the transferrin receptor.

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References

1 Larrick, J.W., and Cresswell, P. (1980) J. Supramol. Struct. 11, 579–586.
2 Trowbridge, I.S. and Omary, M.B. (1981) Proc. Natl. Acad. Sci. USA 78, 3039–3043.
3 Sutherland, R., Delia, D., Schneider, C., Newman, R., Kemshead, J. and Greaves, M. (1981) Proc. Natl. Acad. Sci. USA 78, 4515–4519.
4 Schneider, C., Sutherland, R., Newman, R. and Greaves, M. (1982) J. Biol. Chem. 257, 8516–8522.
5 Goding, J.W. and Burns, G.F. (1981) J. Immunol. 127, 1256–1258.
6 Jing, S. and Trowbridge, I.S. (1987) EMBO J. 6, 327–331.
7 Omary, M.B. and Trowbridge, I.S. (1981) J. Biol. Chem. 256, 12888–12892.
8 Van Driel, I.R. and Goding, J.W. (1985) Eur. J. Biochem. 149, 543–548.
9 Schneider, C., Owen, M.J., Banville, D. and Williams, J.G. (1984) Nature 311, 675–678.
10 McClelland, A., Kuhn, L.C. and Ruddle, F.H. (1984) Cell 39, 267–274.
11 Stearne, P.A., Pietersz, G.A. and Goding, J.W. (1985) J. Immunol. 134, 3474–3479.
12 Ralton, J.E., Jackson, H.J., Zanoni, M. and Gleeson, P.A. (1989) Eur. J. Biochem. 186, 637–647.
13 Reckhow, C.L. and Enns, C.A. (1988) J. Biol. Chem. 263, 7297–7301.
14 Hunt, R.C., Riegler, R. and Davis, A.A. (1989) J. Biol. Chem. 264, 9643–9648.
15 Kohler, G., Howe, S.C. and Milstein, C. (1976) Eur. J. Immunol. 6, 292–295.
16 Laemmli, U.K. (1970) Nature 227, 680–685.
17 Stearne, P.A., Van Driel, I., Grego, B., Simpson, R.J. and Goding, J.W. (1985) J. Immunol. 134, 443–448.
18 Cummings, R.D. and Kornfeld, S. (1982) J. Biol. Chem. 257, 11235–11240.
19 Narasimhan, S., Freed, J.C. and Schachter, H. (1986) Carbohydr. Res. 149, 65–83.
20 Narasimhan, S., Wilson, J.R., Martin, E. and Schachter, H. (1979) Can. J. Biochem. 57, 83–96.
21 Finne, J. and Krusius, T. (1982) Methods Enzymol. 83, 269–277.
22 Cummings, R.D. and Kornfeld, S. (1982) J. Biol. Chem. 257, 11230–11234.
23 Hammarstrom, S., Hammarstrom, M.L., Sundblad, G. Arnarp, J., and Lonngren, J. (1982) Proc. Natl. Acad. Sci. USA 79, 1611–1615.
24 Green, E.D. and Baenziger, J.U. (1987) J. Biol. Chem. 262, 12018–12029.
25 Dahms, N.M. and Hart, G.W. (1986) J. Biol. Chem. 261, 13186–13196.
26 Swiedler, S.J., Freed, J.H., Tarentino, A.L., Plummer, T.H., Jr. and Hart, G.W. (1985) J. Biol. Chem. 260, 4046–4054.
27 Yamashita, K., Ohkura, T., Tachibana, Y., Takasaki, S. and Kobata, A. (1984) J. Biol. Chem. 259, 10834–10840.
28 Santer, U.V., Gilbert, F. and Glick, M.C. (1984) Cancer Res. 44, 3730–3735.
29 Yamashita, K., Tachibana, Y., Ohkura, T. and Kobata, A. (1985) J. Biol. Chem. 260, 3963–3969.
30 Yet, M.-G., Shao, M.-C. and Wold, F. (1988) FASEB J. 2, 22–31.
31 Parekh, R.B., Dwek, R.A., Thomas, J.R., Opdenakker, G. and Rademacher, T.W. (1989) Biochemistry 28, 7644–7662.
32 Parekh, R.B., Tse, A.G.D., Dwek, R.A., Williams, A.F. and Rademacher, T.W. (1987) EMBO J. 6, 1233–1244.