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Isolation and partial characterization of ascites sialoglycoprotein-2 of the cell surface sialomucin complex of 13762 rat mammary adenocarcinoma cells

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Sialomucins are the dominant components of the cell surfaces of some carcinoma ascites cells and have been postulated to inhibit recognition of tumours by the immune system. The sialomucin ASGP-1 (ascites sialoglycoprotein-1) of the 13762 rat mammary adenocarcinoma is associated with the cell surface as a complex with a concanavalin-A-binding glycoprotein called ASGP-2. This sialomucin complex has been purified from ascites cell microvilli by extraction with Triton X-100 and CsCl density-gradient centrifugation. ASGP-1 (which has been purified previously) and ASGP-2 were dissociated in 6 M-guanidine hydrochloride and separated by gel filtration. The molecular mass of the undenatured detergent complex of ASGP-2, estimated by gel filtration and velocity sedimentation in Triton X-100, was 148 kDa. Since the apparent molecular mass by SDS/polyacrylamide-gel electrophoresis was about 120 kDa, ASGP-2 must be a monomer as extracted from the membrane. Studies of its chemical composition indicate that it contains about 45% carbohydrate by weight, including both mannose and galactosamine. Alkaline borohydride treatment of ASGP-2 converted approx. half of the *N*-acetylgalactosamine to *N*-acetylgalactosaminitol, demonstrating the presence of *O*-linked oligosaccharides. Analyses of mannose-labelled Pronase glycopeptides from ASGP-2 by lectin-affinity chromatography on concanavalin A and leucocyte-agglutinating phytohaemagglutinin suggested that 40% of the label was present in high-mannose/hybrid oligosaccharides, 20% in triantennary oligosaccharides substituted on the C-2 and C-4 mannose positions and 40% in tri- or tetra-antennary oligosaccharides substituted on C-2 and C-6. The presence of polylactosamine sequences on these oligosaccharides was suggested by lectin blots and by precipitation from detergent extracts with tomato lectin. From chemical analyses and lectin-affinity studies, we estimate that ASGP-2 contains four high-mannose and 13 complex *N*-glycosylated oligosaccharides, plus small amounts of polylactosamine and *O*-linked oligosaccharides. The presence of four different classes of oligosaccharides on this glycoprotein suggests that it will be an interesting model system for biosynthetic comparisons of the different glycosylation pathways.

INTRODUCTION

Sialomucins are prominent surface constituents of some types of tumour cells (Carraway & Spielman, 1986). Although the details concerning their functions are still unknown, they have been postulated to provide a protective mechanism for tumour cells (Codington & Frim, 1983), masking tumour and histocompatibility antigens (Codington *et al.*, 1978). Moreover, they have been implicated in metastasis (Steck & Nicolson, 1983), and their presence has been shown to be associated with resistance to killing by natural killer cells (Sherblom & Moody, 1986). Studies on monoclonal antibodies directed against human carcinomas have shown that a number of these antibodies react with sialomucins (Ceriani *et al.*, 1983; Magnani *et al.*, 1983; Lan *et al.*, 1985; Miotti *et al.*, 1985; Abe & Kufe, 1986; Davis *et al.*, 1986; Johnson *et al.*, 1986; Schlom, 1986). These monoclonal antibodies may have applications in the diagnosis and therapy of human carcinomas (Schlom, 1986; Ceriani *et al.*, 1987).

In spite of the obvious significance of the sialomucins to carcinomas, very little is known about the details of their structures, their association with membranes and the cell surface or the regulation of their expression. We have been studying ascites sublines of the 13762 rat mammary adenocarcinoma to try to understand some of these parameters (Carraway *et al.*, 1978; Carraway & Spielman, 1986). The sialomucin ascites sialoglycoprotein-1 (ASGP-1) is a major constituent of these ascites cells, comprising $> 0.5\%$ of the total cell protein (Sherblom *et al.*, 1980). In contrast, ASGP-1 is virtually absent when the ascites cells are grown in culture (Howard *et al.*, 1980) or as solid tumours (Huggins *et al.*, 1980). ASGP-1 can be extracted from membranes with guanidine hydrochloride (GdnHCl) or urea and purified on CsCl gradients (Sherblom *et al.*, 1980). It contains about 70% carbohydrate, essentially all as *O*-linked

Abbreviations used: ASGP, ascites sialoglycoprotein; PNA, peanut agglutinin; WGA, wheat-germ agglutinin; ConA, concanavalin A; L-PHA, leucocyte-agglutinating phytohaemagglutinin; PBS, phosphate-buffered saline (30 mM-phosphate/0.15 M-NaCl/2 mM-$MgCl_2$, pH 7.4); LDS, lithium dodecyl sulphate; PAGE, polyacrylamide-gel electrophoresis; PMSF, phenylmethanesulphonyl fluoride; Endo-H, endo-β-*N*-acetylglucosaminidase H; Endo-F, endo-β-*N*-glucosaminidase F; GdnHCl, guanidine hydrochloride.

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oligosaccharide, binds peanut agglutinin (PNA) and has a molecular mass greater than 500 kDa (Sherblom *et al.*, 1980).

The extraction of ASGP-1 from membranes with hydrophilic protein-dissociating agents suggests that it is not an integral membrane protein. When ASGP-1 is extracted from membranes with non-ionic detergent, it is found in the detergent extracts as a 1:1 complex with a second glycoprotein, ASGP-2 (Sherblom & Carraway, 1980). The presence of this complex at the ascites cell surface was confirmed by studies on the redistribution of the components with concanavalin A (ConA) (Helm & Carraway, 1981). ASGP-2 is a smaller sialoglycoprotein, has a lower carbohydrate content (Sherblom & Carraway, 1980) and binds ConA (Sherblom & Carraway, 1980; Helm & Carraway, 1981).

The function of ASGP-2 is unclear. Certainly, its presence stabilizes the association of ASGP-1 with the cell surface. Moreover, a fraction of the cell surface ASGP-2 will associate with the submembrane cytoskeleton, particularly after proteolysis or sialidase treatments which reduce the negative charge on the associated ASGP-1 (Vanderpuye *et al.*, 1988; Sheng *et al.*, 1989). This association appears to be indirect, occurring through interactions with other cell-surface cytoskeleton-associated glycoproteins (Vanderpuye *et al.*, 1988). Preliminary studies have shown that tunicamycin, which inhibits the *N*-glycosylation of ASGP-2, retards expression of ASGP-1 at the cell surface (J. Spielman & K. L. Carraway, unpublished work). These studies, which also indicate that complex formation is an early event in the pathway to the cell surface, suggest that ASGP-2 may be involved in directing the transit of ASGP-1 to the cell surface. Whether ASGP-2 has other functions or is present on cells which do not contain ASGP-1 is as yet unclear. In the present report we describe the purification of ASGP-2 from the sialomucin complex and investigations of its structure. The results indicate that ASGP-2 is a highly glycosylated cell-surface glycoprotein, which contains high-mannose, *N*-glycosylated complex, polylactosamine and *O*-glycosylated oligosaccharides.

EXPERIMENTAL PROCEDURES

Materials

[2,3,4,5-^{3}H]Leucine, [2-^{3}H]mannose, and [1-^{14}C]glucosamine were from ICN Radiochemicals, Irvine, CA, U.S.A. CsCl was from Accurate Chemical, Westbury, NY, U.S.A. GdnHCl (grade I, water-soluble) was obtained from Sigma. The leucocyte-agglutinating phytohaemagglutinin (L-PHA) column was kindly provided and calibrated by Dr. James Ripka, University of Miami. All lectins were from Sigma. All other reagents were of analytical grade.

Cells, cell labelling and preparation of microvilli

The MAT-Cl subline of the 13762 rat mammary adenocarcinoma was maintained by weekly passage as described previously (Carraway *et al.*, 1979). For experiments in which protein was quantified, 20 μCi of [^{3}H]leucine was injected into each of two rats 16 h before they were killed. For analysis of glycoproteins, cells were metabolically labelled with [^{3}H]mannose and/or [^{14}C]glucosamine (20–200 μCi in each of two rats) in the same manner. After metabolic labelling, cells were recovered from the peritoneal cavity and washed two or three times with phosphate-buffered saline (PBS; 30 mM-phosphate/0.15 M-NaCl/2 mM-$MgCl_2$, pH 7.4). Microvilli were prepared from the washed cells as previously described (Carraway *et al.*, 1982).

Purification of ASGP-1–ASGP-2 complex

Microvilli (500 μl, 6–7 mg of protein) were extracted in 3.2 ml of 0.2% Triton X-100/5 mM-glycine/2 mM-EDTA (pH 9.5) for 15 min at room temperature. The extract was loaded on to a two-phase gradient in which the upper phase consisted of 1.0 ml of 0.2% Triton X-100/PBS, pH 7.4, in 4% sucrose and the lower phase consisted of 0.5 ml of 2% SDS/30 mM-imidazole, pH 7.4, in 10% sucrose. The gradient was centrifuged at 100000 ***g*** for 1 h in an SW 50.1 rotor. The upper layer after this centrifugation is enriched in detergent-soluble microvillar proteins, whereas the lower layer is enriched in microfilament-associated microvillar proteins. The upper layer, containing the ASGP-1/ASGP-2 complex and other membrane proteins, was dialysed and concentrated, and applied to a discontinuous CsCl density gradient formed by successively underlaying five CsCl solutions of 1.3, 1.35, 1.40, 1.45 and 1.5 g/ml densities buffered with PBS containing 0.2% Triton X-100. The gradients were centrifuged in an SW 28 rotor at 100000 ***g*** for at least 40 h at 4 °C. After centrifugation, the gradients were fractionated into 4 ml fractions from the bottom using an Isco fractionator. Aliquots were analysed for radioactivity by mixing with 2–5 ml of Ecolume (ICN) and counting on a Beckman LS-3801 scintillation counter. Alternatively, aliquots were dialysed against 0.1% LDS and analysed for protein by SDS/polyacrylamide-gel electrophoresis (PAGE) (8% gel).

Purification of ASGP-2 from complex

The fraction(s) containing ASGP-1–ASGP-2 complex free of lower molecular mass contaminants were pooled from the CsCl density gradient centrifugation step and dialysed/concentrated in a collodion bag apparatus (Schleicher and Schuell) against 6 M-GdnHCl/5 mM-dithiothreitol/0.2% Triton X-100/10 mM-Tris/HCl, pH 8.0 (complex-dissociating buffer). The volume of sample was concentrated to 0.5–1.0 ml in this apparatus, layered on to a column (1.0 cm × 40 cm) of Sepharose CL-2B and eluted with complex-dissociating buffer. Fractions (0.5–0.7 ml) were collected and aliquots were analysed for radioactivity by scintillation counting and/or by SDS/PAGE.

Amino acid analysis of ASGP-2

Purified ASGP-2 recovered from Sepharose CL-2B dissociative chromatography was hydrolysed in 6 M-HCl in an evacuated glass tube for 22 h at 105 °C. Hydrolysate was evaporated in the presence of methanol several times in order to remove any residual traces of acid. Released amino acids were separated on a JEOL amino acid analyser and quantified from response factors obtained from an analytical run of standards.

Carbohydrate compositional analysis of ASGP-2

The carbohydrate composition of purified ASGP-2 was determined by g.l.c. analysis of alditol acetates as described previously (Hull *et al.*, 1984). Briefly, ASGP-2 was hydrolysed, reduced and acetylated before separation

on a 3% ECNNS g.l.c. column. Identification and quantification were accomplished by using elution times and response factors obtained from standard sugars run coincident with analysis of ASGP-2.

Estimation of the molecular mass of ASGP-2

Purified ASGP-2 was co-chromatographed over an Ultra-gel 3A4 column with catalase (240 kDa), IgG (150 kDa) and ovalbumin (45 kDa) in PBS containing 0.2% Triton X-100 to estimate its Stokes radius. The sedimentation coefficient of ASGP-2 was estimated by centrifugation on a 5–20% sucrose-density gradient with catalase, IgG and ovalbumin, using a 5 ml gradient buffered with PBS containing 0.2% Triton X-100 in an SW 50.1 rotor centrifuged at 100000 ***g*** overnight. The molecular mass of ASGP-2 was estimated from the experimentally determined Stokes radius, sedimentation coefficient and partial specific volume as described previously (Liu *et al.*, 1986).

Lectin-affinity chromatography of ASGP-2 glycopeptides

ASGP-2 purified from cells metabolically labelled with [2-^{3}H]mannose was treated exhaustively with a freshly prepared 1% Pronase solution in 10 mM-Tris/HCl/1 mM-$CaCl_2$, pH 8.0, (total vol. 0.5 ml) for 72 h at 45 °C with fresh additions of 1% proteinase solution (0.2 ml) every 24 h. Glycopeptides generated by Pronase digestion were desalted over a Sephadex G-25 column (1 cm × 40 cm) eluted with 7% propan-2-ol. The desalted ASGP-2 glycopeptides were analysed by ConA-affinity chromatography (Cummings & Kornfeld, 1982*a*) by elution over a ConA–agarose (14 mg of ConA/ml of gel) column (0.5 cm × 1.2 cm). Complex glycopeptides of the tri- and tetra-antennary type were eluted from the column using 10 ml of PBS containing 1 mM-$CaCl_2$, 1 mM-$MgCl_2$ and 0.02% NaN_3. Complex glycopeptides of the biantennary type were then eluted from the column using 10 ml of buffer containing 10 mM-methyl α-glucoside. Finally, high-mannose/hybrid-type glycopeptides were eluted from the lectin column using 10 ml of buffer containing 100 mM-methyl α-mannoside. Recovery of the mannose label was 90–95%.

L-PHA lectin affinity chromatography of ConA-unbound complex-type glycopeptides was performed as described by Merkle & Cummings (1987*a*). Briefly, the desalted ConA-unbound fraction in a final volume of 0.3–0.5 ml was layered on to an L-PHA–agarose column (0.3 cm × 30 cm). Fractions (0.25 ml) were collected and aliquots of each fraction were analysed for radioactivity. The unretarded and retarded volumes of the L-PHA column were determined by elution of mannose-labelled BW 5142 mouse lymphoma ConA-unbound complex glycopeptides which have been characterized previously (Cummings & Kornfeld, 1982*b*). Recovery of glycopeptides was 90–95%.

Lectin blot analysis of ASGP-2

Blot analysis of microvillar membrane glycoproteins was accomplished using various lectins of defined specificity. Triton X-100 extracts of microvilli were subjected to electrophoresis on an SDS/8% PAGE gel and transferred to nitrocellulose (Vanderpuye *et al.*, 1988) using a Bio-Rad electroblotting apparatus. The electroblot-transferred proteins were then incubated with specified lectin–peroxidase conjugate solutions and the stain was developed as described previously (Vanderpuye *et al.*, 1988; Sheng *et al.*, 1989).

Lectin precipitations of detergent lysates of microvilli

Microvilli (5 mg/ml of protein) in Tris-buffered saline, pH 7.5, containing 1 mM-$CaCl_2$ were treated for 16 h at 25 °C with or without 0.1 mg of trypsin/ml. Digestion was stopped with a cocktail of proteinase inhibitors containing phenylmethanesulphonyl fluoride (PMSF; 0.3 mM), egg-white trypsin inhibitor (0.2 mg/ml), leupeptin (10 μg/ml), antipain (10 μg/ml), benzamidine (10 μg/ml) and aprotinin (10 μg/ml). Microvilli were washed and lysed with Triton X-100 containing the inhibitors and centrifuged as previously described (Vanderpuye *et al.*, 1988). Aliquots of the Triton supernatants (600 μl) were incubated with increasing concentrations of wheat-germ agglutinin (WGA) and tomato lectin for 1 h at 25 °C and then centrifuged for 2 min at 12000 ***g***. The resulting pellets were washed and prepared for electrophoresis and immunoblotting.

Glycosidase digestions of purified ASGP-2

Purified ASGP-2 was denatured by boiling for 1 min in 0.5% SDS, adjusted to a Triton X-100/SDS ratio of 5:1 and treated with enzyme for 16 h at 37 °C. Conditions for each enzyme are detailed below. After incubation, the samples were boiled for 3 min to denature the enzyme, dialysed against 0.1% LDS and analysed on a SDS/5–15% PAGE gradient gel. Conditions were: 10 munits of Endo F in 50 mM-sodium phosphate, pH 5.5; 10 munits of Endo H in 50 mM-sodium phosphate/2 mM-EDTA, pH 7.4; 10 munits of sialidase in 50 mM-sodium citrate, pH 5.5; 10 munits of endo-β-galactosidase in 50 mM-sodium phosphate, pH 5.5; 10 munits of *N*-Glycanase in 50 mM-sodium phosphate, pH 7.5. Control samples consisted of substrate and buffer without added enzyme. Each digestion mixture was incubated in the presence of a proteinase inhibitor cocktail mixture consisting of PMSF, pepstatin, leupeptin and NaF.

Trifluoromethanesulphonic acid deglycosylation of ASGP-2

Lyophilized ASGP-2 (20 μg) was treated under N_2 with 50 μl of trifluoromethanesulphonic acid at 0 °C for 1 h. The solution was neutralized with 60% (v/v) pyridine/50 mM-ammonium carbonate and dialysed in preparation for SDS/PAGE.

Analytical techniques

SDS/PAGE and immunoblot analyses of proteins were performed as described previously (Sheng *et al.*, 1989) using the electrophoresis methods of King & Laemmli (1971). Antibody directed against ASGP-2 was prepared by immunoblot purification from anti-membrane antiserum (Vanderpuye *et al.*, 1988) or by injection of purified ASGP-2 into rabbits (Sheng *et al.*, 1989). Alkaline borohydride treatment of ASGP-2 was performed as described previously for ASGP-1 (Hull *et al.*, 1984). Analysis of labelled amino sugars was performed after hydrolysis in 4 M-HCl at 105 °C for 6 h. Samples were neutralized, lyophilized and dissolved in 0.01 M-HCl for injection on to a 4 mm × 250 mm column of Aminex A9 (Bio-Rad) maintained at 75 °C and eluted with 88.4 mM-KH_2PO_4/5% methanol/10% acetonitrile, pH 7.0, at 0.25 ml/min (Lohmander, 1986). Fractions of 0.5 ml were collected and analysed for radioactivity. The

column was calibrated with authentic glucosamine, galactosamine and galactosaminitol.

RESULTS

Isolation of ASGP-2

Sialomucin complex (ASGP-1–ASGP-2) was previously observed in detergent extracts of ascites cells or isolated ascites cell microvilli (Sherblom & Carraway, 1980). To minimize contamination from smaller proteins of whole cell extracts (Sherblom & Carraway, 1980) and to reduce the likelihood of proteolysis during isolation, ASGP-2 was isolated from sialomucin complex extracted from ascites microvilli. Cells labelled with [^{14}C]glucosamine and [^{3}H]leucine were used in early experiments to facilitate detection and analysis of the glycoproteins. A four-step isolation procedure was used. (1) Microvilli were isolated by differential centrifugation of cells gently sheared through a 14 gauge syringe needle (Carraway *et al.*, 1982). (2) Microvilli were extracted with Triton X-100 and centrifuged to remove microvillar microfilaments and associated proteins (Carraway *et al.*, 1985). The extraction procedure given in the Experimental procedures section was developed to permit concomitant isolation of ASGP-2 and cytoskeleton-associated proteins and is one of several which have been used for ASGP-2 isolation. (3) Sialomucin complex was isolated by CsCl density-gradient centrifugation of the Triton extract supernatant (Sherblom & Carraway, 1980). As shown in Fig. 1, the CsCl gradient cleanly separates the ASGP-1–ASGP-2 complex, which migrates near the bottom of the gradient due to its high density (Sherblom *et al.*, 1980), from the other Triton-soluble proteins of the microvilli. (4) ASGP-2 was separated from ASGP-1 by dissociative gel-filtration chromatography of the sialomucin complex on Sepharose CL-2B in GdnHCl, as shown in Fig. 2(*a*). SDS/PAGE of the purified ASGP-2 shown in Fig. 2(*b*) indicates > 95% purity. The recovery of ASGP-2 from microvilli was very high (600 μg of ASGP-2 protein from 6.0 mg of microvillar protein), and indicated that it represents at least 10% of total microvillar protein. An alternative method for isolating ASGP-2 involved preparative SDS/PAGE of the purified sialomucin complex and electroelution of ASGP-2 from the gels. This latter procedure was faster and gave comparable yields.

Chemical and physical properties of ASGP-2

From a combination of amino acid analysis and carbohydrate compositional analyses (Table 1), ASGP-2 is about 45% carbohydrate by mass. Aspartic acid, serine, threonine and glycine comprise 43% of the polypeptide. The Gal/Man/GlcNAc ratio of 9:10:15 could result either from a mixture of high-mannose and complex *N*-linked carbohydrate chains or from the presence of complex chains only. The presence of GalNAc suggests the presence of *O*-linked carbohydrate, which was verified by the conversion of approx. half of the *N*-acetylgalactosamine to *N*-acetylgalactosaminitol by alkaline borohydride treatment of purified ASGP-2, as determined by h.l.p.c. analyses of the amino sugars obtained after acid hydrolysis (results not shown). Sedimentation velocity and gel filtration analyses in the presence of detergent yielded a sedimentation coefficient of 6.0 S and a Stokes radius of 4.6 nm (Table 2). From these parameters and the partial specific volume of 0.68 ml/g estimated from the chemical composition, a molecular mass of 148 kDa was calculated. However, this value includes a contribution from detergent bound to the glycoprotein. As shown previously, the apparent molecular mass from SDS/PAGE is 120 kDa (Sherblom & Carraway, 1980). Thus the isolated ASGP-2 molecule is a monomer, and the apparent size of its polypeptide portion is 65–80 kDa, based on these data.

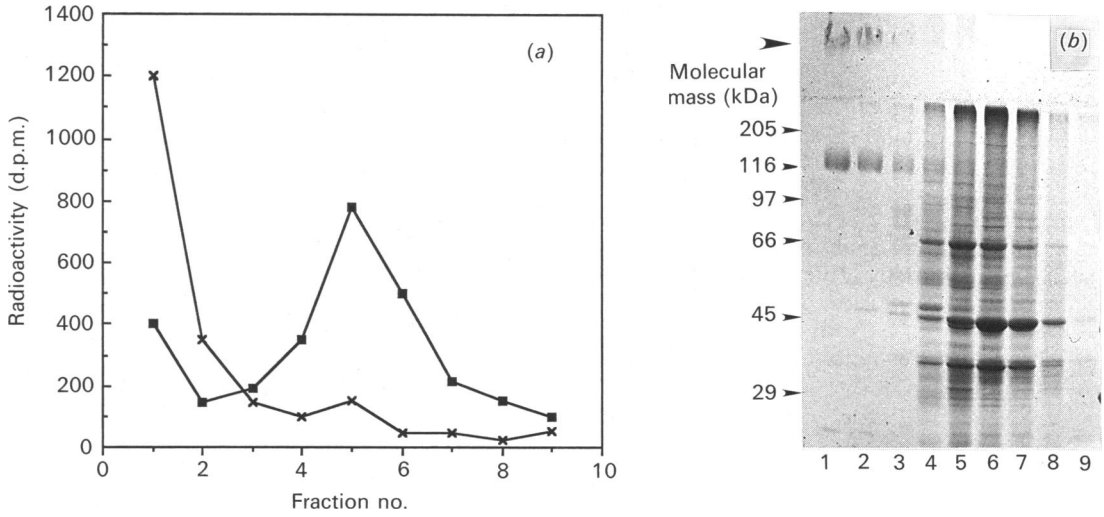

Fig. 1. Isolation of the sialomucin complex from rat mammary adenocarcinoma microvilli

Triton X-100 supernatants (1.2 ml) from microvilli of MAT-Cl ascites cells metabolically radiolabelled with [^{3}H]leucine (■) and [^{14}C]glucosamine (×) (Carraway *et al.*, 1982) were fractionated by CsCl gradient centrifugation as previously described (Sherblom & Carraway, 1980). Aliquots (100 μl) of fractions 1–9 (bottom to top) were analysed for (*a*) radioactivity and (*b*) protein components by SDS/PAGE. The large arrowhead in (*b*) shows the position of migration of ASGP-1 in the stacking gel. Smaller arrowheads indicate the migration of molecular mass standards.

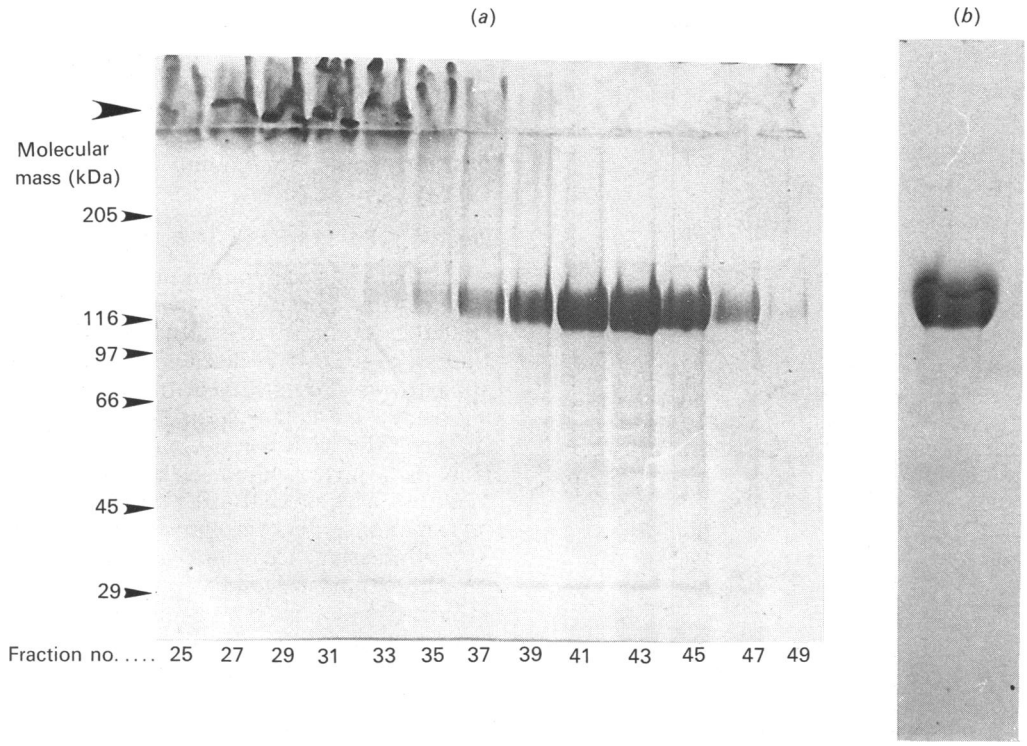

Fig. 2. Isolation of ASGP-2 from the glycoprotein complex

The purified complex from density-gradient centrifugation was dialysed against complex-dissociating buffer (6 M GdnHCl/5 mM-dithiothreitol/0.2% Triton X-100/10 mM-Tris/HCl, pH 8.0) and applied to a column of Sepharose CL-2B (1 cm × 40 cm) eluted with complex-dissociating buffer. Fractions (600 μl) were collected and appropriate aliquots were analysed for (*a*) protein components by SDS/PAGE and Coomassie Blue staining. Fractions 41–45 containing ASGP-2 were pooled, and an aliquot containing approx. 10 μg was analysed by SDS/PAGE and Coomassie Blue staining to verify purity (*b*). Migration of molecular mass standards and ASGP-1 are shown as in Fig. 1.

Table 1. Chemical composition of ASGP-2

Amino acid/sugar	Composition (μg/100 μg wt.)	Composition (mol/100 mol of amino groups)
Asx	7.0	12.0
Thr	3.8	7.2
Ser	5.1	11.0
Glx	5.4	8.2
Pro	2.6	5.1
Gly	4.1	12.3
Ala	2.8	7.2
Cys	1.5	2.8
Val	1.9	3.7
Met	0.4	0.7
Ile	1.9	3.3
Leu	5.0	8.6
Tyr	2.3	2.9
Phe	3.5	4.8
His	1.9	2.7
Lys	2.3	3.5
Arg	3.0	3.9
Total protein	54.5%	
Gal	8.9	11.2
Man	9.9	12.4
GlcNAc	18.1	18.6
GalNAc	1.3	1.4
NeuAc	7.0	5.1
Total carbohydrate	45.2%	

Table 2. Physical properties of ASGP-2

Stokes radius	4.6 nm
Sedimentation coefficient	6.0 S
Frictional ratio	1.35
Partial specific volume	0.68 ml/g
Molecular mass (native, detergent complex)	148 kDa
Molecular mass (SDS/PAGE)	120 kDa

ASGP-2 glycopeptides

To obtain information on the structural features of the *N*-linked oligosaccharides of ASGP-2, ConA lectin-affinity chromatography of [^{3}H]mannose glycopeptides (Fig. 3*a*) was performed. Of the mannose label applied to the column, 60% was recovered as unbound glycopeptide, diagnostic of tri- and tetra-antennary complex oligosaccharides (Cummings & Kornfeld, 1982*b*). Negligible amounts of mannose label were recovered from the fraction eluted with 10 mM-methyl α-glucoside (biantennary and bisected complex structures). The remaining mannose label (40%) was recovered by elution with 100 mM-methyl α-mannoside (high-mannose and hybrid glycopeptides). Preliminary evidence from h.p.l.c. analyses of oligosaccharides obtained by Endo H treatment of these ConA-bound glycopeptides indicated that they consisted predominantly of Man_{6-7} high-mannose structures (results not shown).

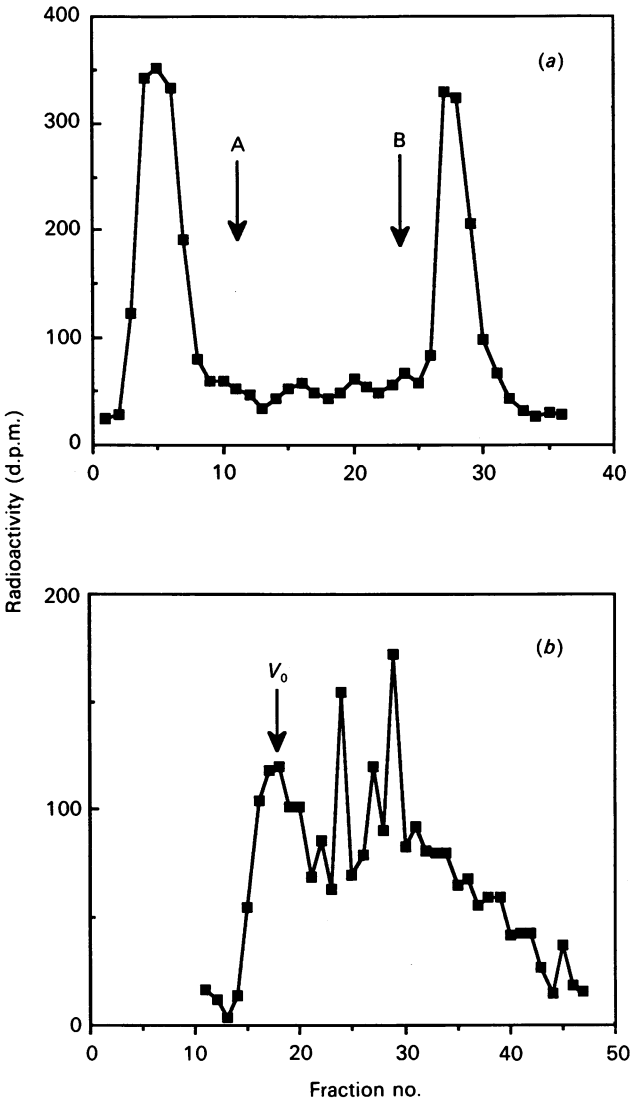

Fig. 3. Lectin-affinity chromatography of ASGP-2 glycopeptides

(*a*) [^{3}H]Mannose-labelled ASGP-2 glycopeptides were layered on to a 1.2 ml column of ConA–agarose eluted with PBS/1 mM-$CaCl_2$/1 mM-$MgCl_2$, pH 7.4. The unbound fractions (1–11) were eluted with buffer alone. The weakly bound fractions (12–24) were eluted with buffer containing 10 mM-methyl α-glucoside (arrow A). The strongly bound fractions (25–36) were eluted with buffer containing 100 mM-methyl α-mannoside (arrow B). (*b*) The [^{3}H]-mannose-labelled ASGP-2 ConA-unbound glycopeptide peak was further analysed on an L-PHA-Sepharose column (0.4 cm × 30 cm) eluted with PBS, pH 7.4. Fractions of 0.3 ml were collected. From calibration experiments using BW 5142 mouse lymphoma glycopeptides, the unretarded fractions (15–21) and the retarded fractions (22–40) were identified.

Further characterization of the complex ASGP-2 glycopeptides (ConA-unbound) was achieved by L-PHA lectin-affinity chromatography (Fig. 3*b*). L-PHA binds specifically to *N*-acetyl-lactosamine linked to the 6-position of a mannose core residue (Cummings & Kornfeld, 1982*a*). Of the mannose label applied to the column, 30% was not retarded by the lectin, suggesting that 30% of the complex fraction consists of triantennary oligosaccharides with outer mannose residues substituted in the C-2 and C-4 positions with lactosamine. The remaining mannose label (70%) was retarded by the lectin, indicating tri- and/or tetra-antennary chains with outer mannose residues substituted in the C-2 and C-6 position with lactosamine (Cummings & Kornfeld, 1982*a*).

Deglycosylation of ASGP-2

Further characterization of the ASGP-2 structure was obtained by digestion of the purified glycoprotein with endoglycosidases followed by SDS/PAGE analysis. Both Endo F and Endo H digestions (Fig. 4) caused a shift in apparent molecular mass from 120 kDa to 100 kDa, consistent with the ConA glycopeptide-affinity data showing the absence of biantennary oligosaccharides (Fig. 3*a*). Since Endo F cleaves biantennary and high-mannose oligosaccharides whereas Endo H cleaves only high-mannose oligosaccharides, they would be expected to yield the same cleavage results for ASGP-2, which has no biantennary oligosaccharides.

Sialidase and endo-β-galactosidase digestions resulted in no apparent change in molecular mass as monitored by SDS/PAGE (results not shown). Since the composition and lectin-binding data suggest the presence of both sialic acid residues and polylactosamine

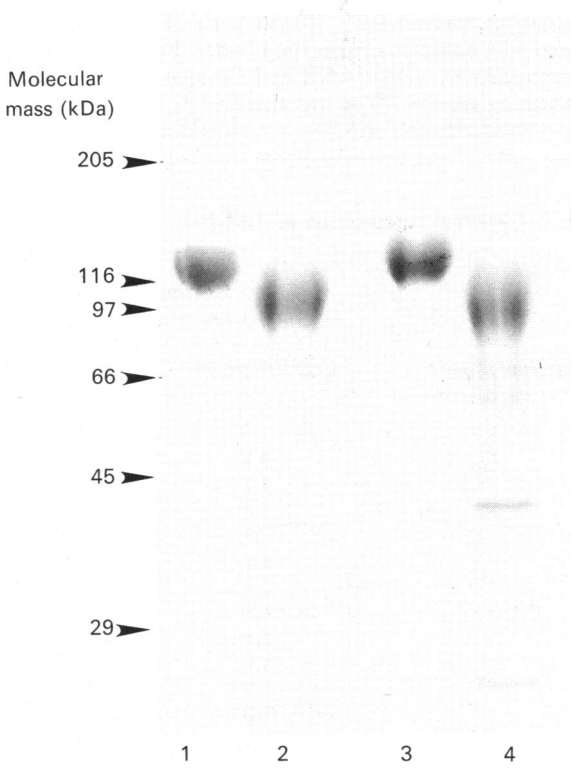

Fig. 4. Endoglycosidase digestions of ASGP-2

ASGP-2 was denatured in 0.5% SDS, adjusted to a 5:1 ratio of Triton X-100/SDS, treated with Endo H and Endo F in the presence of proteinase inhibitors as described in the Experimental procedures section and analysed on an SDS/5–15% PAGE gradient gel with Coomassie Blue staining. Lane 1, ASGP-2 without Endo F added; lane 2, ASGP-2 with Endo F; lane 3, ASGP-2 without Endo H added; lane 4, ASGP-2 with Endo H.

oligosaccharides, the lack of effect of these enzymes may indicate that the amounts of these components are too small to make a detectable contribution to the mobility on SDS/PAGE, or that the structures are altered in some way to make them resistant to the enzyme digestions.

N-Glycanase digestion of ASGP-2 caused a shift in apparent molecular mass from 120 kDa to 70 kDa (Fig. 5*a*). Although the digestion product failed to bind ConA, suggesting that its high-mannose oligosaccharides had been removed as expected, it did react with L-PHA. Thus a fraction of the ASGP-2 complex oligosaccharides were resistant to *N*-Glycanase digestion under the conditions used. When ASGP-2 was deglycosylated with trifluoromethanesulphonic acid, a major product of 60 kDa was produced, which did not bind ConA or L-PHA (Fig. 5*b*). The size change on deglycosylation is consistent with compositional data showing that almost 50% of the ASGP-2 molecule is carbohydrate and indicates that the molecular mass of the glycoprotein is nearer the SDS/PAGE value of 120 kDa than the value obtained for the native glycoprotein in detergent.

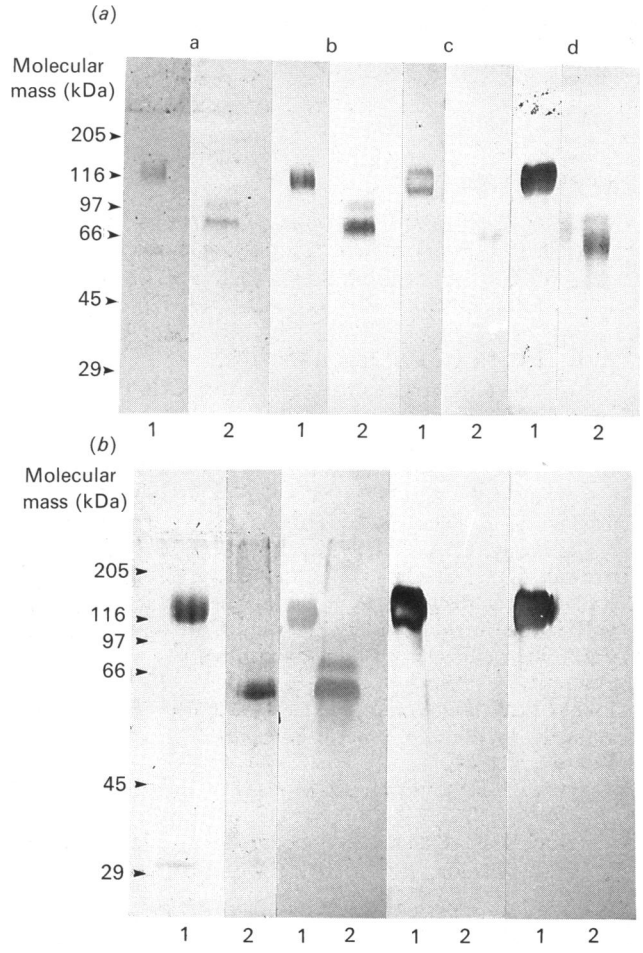

Fig. 5. Deglycosylation of ASGP-2 with *N*-Glycanase and trifluoromethanesulphonic acid

ASGP-2 was prepared for digestion with *N*-Glycanase (*a*) as described for Fig. 4 and treated with trifluoromethanesulphonic acid (*b*) as described in the Experimental procedures section. a, Coomassie Blue stain; b, anti-ASGP-2 immunoblot; c, ConA blot; d, L-PHA blot. Lanes 1, untreated ASGP-2; lanes 2, treated ASGP-2.

Lectin-binding properties of ASGP-2

Additional information on the oligosaccharide composition was obtained from lectin blot analysis of Triton X-100 extracts of microvilli analysed by SDS/PAGE (Table 3). ASGP-2 does not show reaction with PNA as does ASGP-1, indicating the absence of unsubstituted *O*-linked disaccharide moieties (Galβ1,3GalNAc) on ASGP-2. As shown previously, ASGP-2 reacts strongly with ConA, consistent with the presence of high-mannose/hybrid oligosaccharides (Fig. 3*a*). Both ASGP-1 and ASGP-2 show strong affinity for WGA, which interacts with sialic acid as well as glucosamine. Furthermore, ASGP-2 shows a strong affinity for *Datura* and tomato lectins, suggesting that ASGP-2 carries some

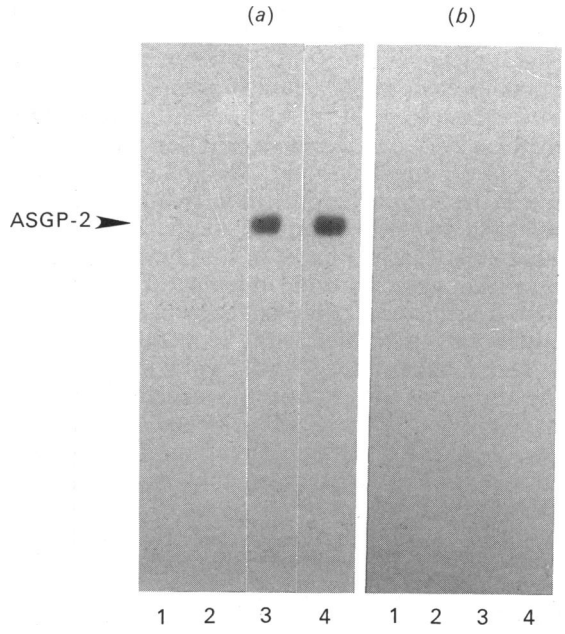

Fig. 6. Tomato lectin precipitation of ASGP-2

Microvilli were incubated with or without trypsin, lysed in detergent and precipitated with various amounts of tomato lectin as described in the Experimental procedures section. The resulting precipitates were collected by centrifugation at 2000 *g* for 10 min and prepared for SDS/PAGE and immunoblotting as described previously (Vanderpuye *et al.*, 1988). (*a*) Anti-ASGP-2 immunoblots of lectin precipitates from trypsin-treated microvilli; (*b*) anti-ASGP-2 immunoblots of lectin precipitates from untreated microvilli. Lanes 1–4 are samples from detergent lysates treated with 0, 10, 50 and 100 μg of tomato lectin respectively.

Table 3. Lectin affinities of ASGP-1 and ASGP-2

Lectin	ASGP-1	ASGP-2	Lectin specificity
PNA	+	–	Galβ1, 3GalNAc
ConA	–	+	βMan, βGlc
WGA	+	+	GlcNAc, sialic
Tomato	–	+	Polylactosamine
L-PHA	–	+	Galβ1, 4GlcNAcβ1, 6Man
Datura	–	+	Polylactosamine
Pokeweed	–	+	Polylactosamine

polylactosamine groups on its complex or *O*-linked oligosaccharides (Merkle & Cummings, 1987*b*).

Additional evidence for the presence of polylactosamine groups was obtained by tomato lectin precipitation of ASGP-2 from detergent lysates (Fig. 6). Interestingly, the precipitation was effective only on microvilli which had been treated with trypsin. Since trypsin cleaves and releases most of the ASGP-1 from the microvilli (Vanderpuye *et al.*, 1988), these results suggest that the association of ASGP-1 with ASGP-2 inhibits lectin binding or cross-linking of the soluble ASGP-2. A similar effect is observed with WGA, which precipitates ASGP-2 from lysates of trypsin-treated, but not untreated, microvilli (results not shown).

DISCUSSION

The 13762 rat mammary adenocarcinoma is a highly malignant metastatic tumour. Sialomucin complex is the major component of plasma membranes of ascites 13762 tumour cells and appears to provide a mechanism by which these cells can avoid recognition by the immune system (Sherblom & Moody, 1986). In microvilli from these cells the complex is present in greater amounts than the microfilament core protein, actin. The abundance, size and high negative charge of the complex dictate that it must play a significant role in regulating interactions at the tumour cell surface. The sialomucin itself appears to be only loosely associated with the membrane bilayer, if at all. The stability of the association of the complex with the membrane must reside in the structure of ASGP-2. We have previously presented evidence from proteolysis studies of ASGP-2 topography that it is an integral membrane protein which probably has a short cytoplasmic domain (Sheng *et al.*, 1989). These proteolysis studies, combined with the present studies of ASGP-2 carbohydrates, indicate that most of the rest of the molecule must be glycosylated.

ASGP-2 is unusual in the number of types of oligosaccharides it contains. From the composition and ConA affinity data, assuming a polypeptide molecular mass of 55 kDa, we estimate that ASGP-2 contains four high-mannose and 13 complex *N*-linked oligosaccharides. The composition and L-PHA binding data indicate the presence of both tri- and tetra-antennary oligosaccharides, but the amounts of galactose and sialic acid suggest that many of these are incomplete. The data are also consistent with a small number of *O*-linked (3–4) and polylactosamine oligosaccharides. These results suggest that ASGP-2 could provide a useful model for comparative analyses of the synthetic routes of the different oligosaccharides. The occurrence of both *N*- and *O*-linked oligosaccharides provides a model for direct analyses of the temporal aspects of these pathways on a cell-surface glycoprotein where both types of oligosaccharides are present.

The presence of both high-mannose and tri- or tetra-antennary oligosaccharides suggests that different sites on ASGP-2 are differentially resistant to processing by mannosidases. Likewise, the high content of L-PHA-binding oligosaccharides indicates the presence in these cells of glucosaminyltransferase V, the enzyme responsible for adding the β1,6-GlcNAc branch to the trimannose core (Yamashita *et al.*, 1985). This enzyme and L-PHA binding have been implicated in metastasis (Dennis *et al.*, 1987), but the significance of these observations is unclear. ASGP-2 is the predominant L-PHA-binding glycoprotein in the 13762 ascites cells (Sheng *et al.*, 1989). High-mannose oligosaccharides are postulated to be a recognition site for natural killer cells (Dennis & Laferte, 1985; Ahrens & Ankel, 1987) and would make ASGP-2 a target for ascites cell destruction. However, these sites may well be masked by the presence of ASGP-1. The ability of ASGP-1 to affect interactions of ASGP-2 with other proteins is documented by the tomato lectin precipitation studies, in which ASGP-2 is precipitated only after ASGP-1 has been cleaved by proteolysis. A similar effect is observed with ASGP-2 association with microvillar microfilament cores, which is enhanced after proteolytic cleavage of ASGP-1 (Vanderpuye *et al.*, 1988). These observations provide support for the proposed role of ASGP-1 as an inhibitor of cell-surface recognition processes (Carraway *et al.*, 1988).

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