Sulfate Incorporation into the Major Sialoglycoprotein of the MAT-B1 Subline of the 13762 Rat Ascites Mammary Adenocarcinoma†

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ABSTRACT: MAT-B1 and MAT-C1 sublines of the 13762 rat mammary adenocarcinoma differ substantially in agglutinability, mobility of lectin receptors, and xenotransplantability. When these sublines are metabolically labeled with [^{3}H]-glucosamine and [^{35}S]sulfate, both show a predominant, ^{3}H-labeled glycoprotein component, termed ASGP-1, which migrates slowly on dodecyl sulfate–polyacrylamide gels. ASGP-1 from MAT-B1 cells is also labeled by [^{35}S]SO_4^{2-}, but MAT-C1 ASGP-1 is not. Papain digestion followed by DEAE-cellulose chromatography indicates that neither of the two sublines incorporates significant amounts of [^{3}H]glucosamine into cellular glycosaminoglycan. MAT-B1 ASGP-1 was isolated for analysis of its sulfated oligosaccharides by centrifugation of MAT-B1 membrane fragments in CsCl containing 4 M guanidine hydrochloride. Refractionation on a similar gradient under isopycnic conditions yielded fractions in which increases in ^{35}S/^{3}H ratios, carbohydrate/protein ratios, and ratios of *N*-acetylglucosamine (GlcNAc), fucose, and sialic acid to *N*-acetylgalactosamine (GalNAc) were found with increasing density. Oligosaccharides of doubly labeled MAT-B1 ASGP-1 were released by treatment with alkaline borohydride and fractionated by gel filtration. Five major peaks, overlapping but not coincident, were found for ^{3}H and ^{35}S labels. The separated oligosaccharides were further fractionated by ion-exchange chromatography for carbohydrate analysis, which showed that >50% of the oligosaccharides are neutral (based on recovery of *N*-acetylgalactosaminitol) and that the major component is the disaccharide containing galactose (Gal) and GalNAc. About 20% of the oligosaccharides contain sulfate, about 30% contain sialic acid, and about 30% have significant amounts of fucose. At least one oligosaccharide containing both sulfate and sialic acid (SA) was found in significant amounts (5% of total, composition GalNAc/GlcNAc/Gal/SA/Fuc 1:1:1:2:0). One of the major sulfated oligosaccharides contains sulfate and fucose (7% of total, composition GalNAc/GlcNAc/Gal/SA/Fuc 1:1:1:0:1). Tentative structural assignments can be made for the major oligosaccharides based on the compositional analyses.

Mucin-type glycoproteins, which have O-linked oligosaccharides, are found normally both in epithelial secretions and associated with cell surface membranes (Horowitz & Pigman, 1977; Hughes, 1976). The function(s) of the membrane glycoproteins is largely obscure. One possibility is that they serve in recognition functions, exemplified by the fact that they bear the MN blood group determinants in erythrocytes (Winzler, 1972) and other cells (Springer et al., 1972; Codington et al., 1975). Alternatively, the mucin-type glycoproteins may serve a protective role for the cell. It has been suggested that the major sialoglycoprotein (epiglycanin) of the allotransplantable TA3-Ha mouse mammary adenocarcinoma subline aids the escape of these tumor cells from immune surveillance of the host, either by directly masking histocompatibility antigens at the cell surface (Sanford et al., 1973; Miller et al., 1977) or by being shed from the cell surface in a form which participates in "blocking" the immune response to the tumor (Cooper et al., 1974). Interestingly, the disaccharide precursor (Gal-GalNAc)[1] of the MN blood group determinants (Springer et al., 1976) is detected by immunological techniques in epiglycanin (Codington et al., 1972) and in malignant human breast tumors but not in benign breast lesions (Springer et al., 1975).

We have been using the 13762 rat mammary adenocarcinoma to investigate the expression and possible functions of a major cell surface mucin-type sialoglycoprotein, termed ASGP-1. Two ascites sublines of this tumor (MAT-B1 and MAT-C1) differ substantially in agglutinability, lectin receptor mobility, morphology, and xenotransplantability (Carraway et al., 1978; Buck et al., 1979). However, unlike TA3-Ha and TA3-St sublines of the TA3 tumor, which differ in xenotransplantability and in their content of epiglycanin (Miller et al., 1977), MAT-B1 and MAT-C1 cells have approximately equivalent amounts of ASGP-1. ASGP-1 can be purified to homogeneity from either subline (Sherblom et al., 1979). The molecular weights in 4 M Gdn·HCl (MAT-B1, 570 K; MAT-C1, 690 K) and carbohydrate contents (MAT-B1, 67%; MAT-C1, 73%) of the glycoproteins from the two sublines are similar, and the amino acid compositions are nearly identical. However, substantial differences were found in the carbohydrate compositions and size distributions of oligosaccharides released by alkaline borohydride treatment (Sherblom et al., 1980).

To explore further the cell surface differences between MAT-B1 and MAT-C1, we have investigated sulfate incorporation into these sublines. Both synthesize small amounts of sulfated glycosaminoglycans, but only in MAT-B1 cells is the major portion of the sulfate incorporated into ASGP-1. The oligosaccharides from ASGP-1 have been fractionated by sequential Bio-Gel P-4 and DEAE-Sephadex chromatog-

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[1] Abbreviations used: PBS, Dulbecco's phosphate-buffered saline, pH 7.4, without calcium or magnesium; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; $NaDodSO_4$, sodium dodecyl sulfate; Gdn·HCl, guanidine hydrochloride; GalNAc, *N*-acetylgalactosamine; GalNAcOH, *N*-acetylgalactosaminitol; GlcNAc, *N*-acetylglucosamine; Gal, galactose; SA, sialic acid; Fuc, fucose.

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raphy for determination of the distribution of sulfate. About 20% of the oligosaccharides were found to be sulfated, and both sulfate and sialic acid were detected on some oligosaccharides.

Experimental Procedures

Materials. D-[1-^{3}H]Glucosamine (2–6 Ci/mmol) and sodium [^{35}S]sulfate (100 mCi/mmol) were from Amersham; Instagel was from Packard; CsCl (99.99%) was a product of Varlacoid; guanidine hydrochloride (Grade I) was from Sigma. Carbohydrate standards included inositol (Supelco), *N*-acetylglucosamine, *N*-acetylgalactosamine, glucose, galactose, and *N*-acetylneuraminic acid (Sigma), mannose (K & K Laboratories), and fucose (Pfanstiehl Laboratories); *N*-acetylgalactosaminitol was prepared by reduction of *N*-acetylgalactosamine (Laine et al., 1972).

Cells. MAT-B and MAT-C ascites sublines of the 13762 mammary tumor were obtained from Mason Research Laboratories and were maintained as described previously (Carraway et al., 1976). MAT-B1 and MAT-C1 designate the sublines which arose from the MAT-B and MAT-C lines, respectively, by routine weekly passage for ~1 year in our laboratory (Carraway et al., 1978). The distinguishing features of these sublines have been stable for over 2 years since the original observations. Cells are passed by weekly transfers and maintained as frozen stocks. Cells used for these studies were washed 3 times with cold PBS after recovery from the peritoneal cavity.

Cell Labeling. Metabolic labeling with [^{3}H]glucosamine (100 μCi) and [^{35}S]sulfate (1 mCi) was accomplished by injection of the mixture in 0.5 mL of 0.9% NaCl into the peritoneal cavity of a tumor-bearing rat ~16 h prior to sacrifice and recovery of the cells. Typically, greater than 70% of the ^{3}H of washed cells is associated with the major glycoprotein (Sherblom et al., 1980).

Extraction and Analysis of Glycosaminoglycans. Approximately 1×10^9 cells were incubated with 10 mL of acetone (30 min, room temperature), 10 mL of 3:1 ether–acetone (60 min, 37 °C), and 10 mL of ether (30 min, room temperature), and the residue was collected by centrifugation at 1000*g* for 5 min. The dried residue was mixed with 1 to 2 mg of papain in 2 mL of 0.06 M sodium acetate buffer, pH 6.0, with 0.05 M EDTA and 0.01 M cysteine and incubated at 60 °C for 4 h. The digest was made 10% in trichloroacetic acid, allowed to stand on ice for 60 min, and centrifuged at 10000*g* for 20 min. The supernatant was dialyzed at 4 °C against 0.02 M sodium acetate, pH 5.0, and 0.01 M NaCl.

This extract was applied to a 0.9 × 5 cm column of DEAE-cellulose (Whatman DE-52), washed with 0.02 M sodium acetate, pH 5.0, and 0.01 M NaCl, and eluted with a 500-mL linear gradient of 0.01–1.0 M NaCl in 0.02 M sodium acetate, pH 5.0.

Gel Electrophoresis. $NaDodSO_4$–polyacrylamide gel electrophoresis was performed as described by King & Laemmli (1971) with a 5% separating gel and a 3% stacking gel.

Radioactivity Determination. Aliquots of whole cells, membranes, or gradient or column fractions were mixed with 3 mL of Instagel and counted in a Packard Tri-Carb scintillation counter. The ^{3}H counts were corrected for ^{35}S spillover when both isotopes were used. Polyacrylamide gel slices (1.5 mm) were incubated 1 h at 60 °C with 0.2 mL of Soluene 350, mixed with 3 mL of Instagel, and placed in the dark for at least 6 h prior to counting.

Isolation of ASGP-1 from Membrane Fragments. Washed cells were suspended in 10 volumes of 10 mM Tris, pH 8.0, and allowed to stand on ice for 2 min prior to centrifugation at 600*g* for 2 min. The swollen cell pellet was suspended in 10 volumes of 10 mM Tris, pH 8.0, and homogenized by four to five strokes of a Dounce homogenizer with a tight pestle. Immediately after homogenization the suspension was brought to a concentration of 3 mM in Mg^{2+} by addition of 30 mM $MgCl_2$ and 100 mM NaCl. The homogenate was centrifuged at 1000*g* for 1 min, and the supernatant was centrifuged at 10000*g* for 10 min. Membrane fragments were collected by centrifugation of the supernatant at 100000*g* for 90 min in an SW27 rotor. Preformed CsCl gradients were prepared by gently layering under each other 2 mL each of 4 M Gdn·HCl and 10 mM Tris, pH 7.4, containing 1.58, 2.0, 2.37, 2.79, and 3.15 M CsCl in 5/8 × 3 in. centrifuge tubes. Membrane fragments were suspended in 10 mM Tris, pH 8.0, with a hand homogenizer and made 4 M in Gdn·HCl by addition of solid Gdn·HCl. The mixture was vortexed vigorously, 1 mL (~5 mg of membrane protein) was layered onto the CsCl gradient, and the gradients were centrifuged in a Beckman 75 Ti rotor at 4 °C for 24 h at 55000 rpm. The density of selected fractions was determined by the difference in the dry and filled weight of a 100-μL glass constriction pipet calibrated with distilled water. Fractions were pooled as indicated in Figure 3 for ASGP-1. CsCl and Gdn·HCl were removed by dialysis/concentration with a collodion bag apparatus.

Isopycnic Centrifugation. MAT-B1 cells which had been labeled with [^{3}H]glucosamine and [^{35}S]SO_4^{2-} were used to prepare ASGP-1. Fractions containing ASGP-1 from the density gradient were mixed with 2.37 M CsCl, 4 M Gdn·HCl, and 10 mM Tris, pH 7.4, in a 5/8 in. × 3 in. centrifuge tube, centrifuged at 4 °C for 40 h at 55000 rpm in a Ti 75 rotor, and fractionated as described above. Various fractions were pooled and dialyzed/concentrated into distilled water.

Fractionation of Oligosaccharides. A sample of ASGP-1 (Figure 3) which had been dialyzed against distilled water was incubated at 45 °C with 1.0 M $NaBH_4$ and 0.05 M NaOH for 16 h and neutralized with acetic acid. The mixture was chromatographed on Bio-Gel P-4 (200–400 mesh) equilibrated with 0.1 M pyridine acetate, pH 6.0. The column was calibrated with stachyose, raffinose, maltose, and galactose. Oligomers of hyaluronic acid (umbilical cord), obtained by digestion with hyaluronidase (Hascall & Heinegard, 1974), provided additional calibration.

Fractions designated in Figure 4 were lyophilized, suspended in 0.05 M pyridine acetate, pH 5.0, and applied to a 1.5 × 20 cm column of DEAE-Sephadex. The column was washed with the same buffer and eluted with a 240-mL linear gradient from 0.05 to 0.65 M pyridine acetate, pH 5.0.

Thin-layer chromatography (silica gel G, chloroform–methanol 9:1) was performed on oligosaccharides which had been incubated with acetic anhydride–pyridine (1:1) at room temperature for 40 h. Sections of gel (1 cm) were scraped from the plate for scintillation counting.

Carbohydrate Analysis. Aliquots of ASGP-1 fractions or oligosaccharides (Figure 5) were lyophilized in borosilicate ampules and hydrolyzed with 0.5 mL of 0.5 M methanolic HCl for 16 h at 65 °C under N_2. Reacetylation with acetic anhydride, de-O-acetylation with methanolic HCl, trimethylsilyl derivatization, extraction with hexane, and gas–liquid chromatography were performed by the methods of Reinhold (1972) with inositol as the internal standard.

Results

Glycosaminoglycan Analysis. Previous studies have shown that [^{3}H]glucosamine is incorporated predominantly into ASGP-1 in both MAT-B1 and MAT-C1 cells (Sherblom et

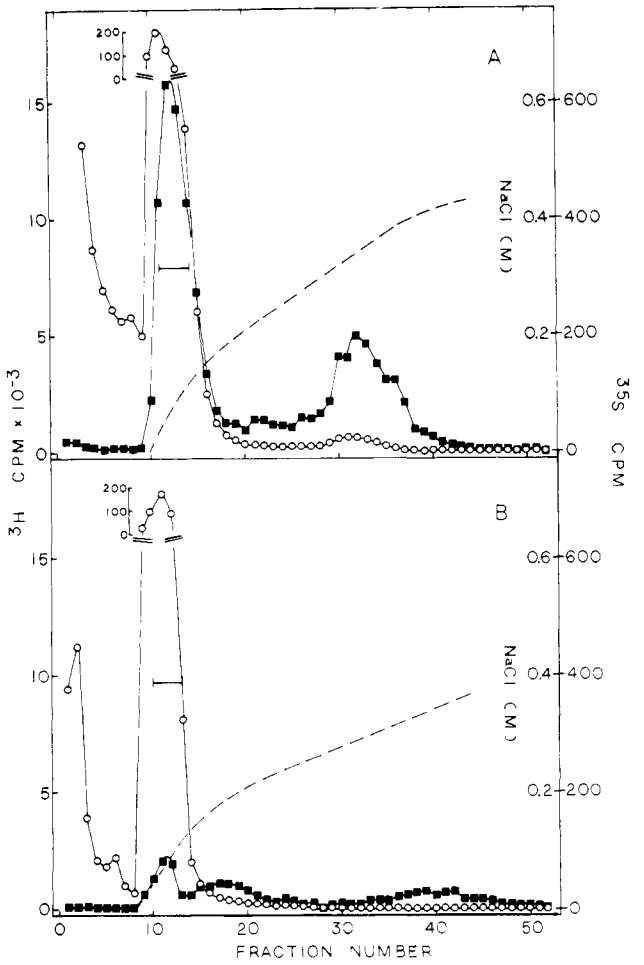

FIGURE 1: DEAE-cellulose chromatography of glycosaminoglycan extract from (A) MAT-B1 and (B) MAT-C1 cells. Cells were labeled metabolically with [^{3}H]glucosamine and [^{35}S]SO_4^{2-}, and glycosaminoglycan extracts were analyzed as described under Experimental Procedures. The sample (1.5 mL) was applied to a 0.9 × 5 cm column of DEAE-cellulose (Whatman DE-52) equilibrated with 0.01 M NaCl and 0.02 M sodium acetate, pH 5.0, washed with 150–200 mL of buffer, and eluted with a 500-mL linear gradient from 0.01 to 1.0 M NaCl. The conductance of selected fractions was measured to determine the NaCl concentration (---). An aliquot of each fraction was removed for determination of ^{3}H (○) and ^{35}S (■) radioactivity. Fractions designated by a bar were pooled to constitute the "glycopeptide" sample.

al., 1980). Labeled, washed MAT-B1 and MAT-C1 cells had 170 and 150 cpm/μg of protein, respectively, after a 16-h labeling period. Labeling with [^{35}S]sulfate for 16 h gave 1.6 and 2.6 cpm/μg of protein for MAT-B1 and MAT-C1 cells, respectively. Virtually all of the glucosamine label (70–90%) from both sublines was recovered in the "glycosaminoglycan extract" prepared by papain digestion. In contrast, only 25% of the sulfate label was obtained in the extract of MAT-C1 cells, compared with a 70% recovery for MAT-B1 cells. Similar results were obtained by using 4 and 24 h of labeling, although the specific activities of both ^{3}H and ^{35}S for cells decreased slightly with increased labeling times. The relative recovery of the two labels in the extracts and fractionation profiles of the extracts on DEAE-cellulose were similar for each time point.

DEAE chromatography of the extract (Figure 1) revealed that 80% of the label from [^{3}H]glucosamine was associated with glycopeptides, defined by their elution at an NaCl concentration of 0.10 M. Greater than 60% of the ^{35}S in the MAT-B1 extract also appeared associated with the glycopeptide peak. A second peak, eluting at 0.35 M NaCl, comprised 30% of the ^{35}S but less than 1% of the ^{3}H in the glycosaminoglycan extract from MAT-B1 cells. No further material eluted when the NaCl concentration was raised to 0.8 M. The results indicate that no hyaluronic acid can be detected in the ascites cells and that a sulfated macromolecule which elutes from DEAE-cellulose at 0.35 M NaCl is a minor component in MAT-B1 cells. Only minor amounts of sulfate label were found in discrete peaks in the MAT-C1 extract. Label from [^{3}H]glucosamine appears mostly associated with glycopeptide which, in the case of MAT-B1 cells, appears to be sulfated.

Since >70% of the [^{3}H]glucosamine incorporated by MAT-B1 and MAT-C1 cells is associated with ASGP-1 (Sherblom et al., 1980), we investigated whether the glycopeptide peak in the DEAE-cellulose profiles might arise from this glycoprotein. First, the amount of ^{3}H associated with sialic acid was determined by chromatography on Bio-Gel P-4 following hydrolysis (0.05 M H_2SO_4, 80 °C, 1 h) under conditions which are relatively specific for cleavage of sialic acid (Warren, 1959). Values of 19% (glycopeptide) and 17% (ASGP-1) were obtained for MAT-B1 and 35% (glycopeptide) and 41% (ASGP-1) for MAT-C1. Second, when glycoprotein samples treated with alkaline borohydride were chromatographed on Bio-Gel P-4, the ^{3}H profiles of the oligosaccharides were essentially the same as those previously reported from alkaline borohydride treatments of purified ASGP-1 from the respective sublines (Sherblom et al., 1980). Moreover, sulfated components appeared to be released by alkaline borohydride treatment of the MAT-B1 glycopeptide, although these components were not coincident with the ^{3}H peaks. No discrete sulfated species were detected in the MAT-C1 glycopeptide (data not shown).

Isolation of Sulfated MAT-B1 ASGP-1. We have previously identified ASGP-1 by its (1) low electrophoretic mobility on NaDodSO$_4$–polyacrylamide gels and (2) density of 1.4 g/cm^3 in a CsCl gradient containing 4 M Gdn·HCl. To establish whether MAT-B1 ASGP-1 was sulfated, we subjected [^{3}H]glucosamine- and [^{35}S]SO_4^{2-}-labeled cells to NaDodSO$_4$–polyacrylamide gel electrophoresis and to CsCl density gradient centrifugation in the presence of 4 M Gdn·HCl. On NaDodSO$_4$ gels a distinct ^{35}S peak appeared in the region of low electrophoretic mobility in MAT-B1 cells (Figure 2A). MAT-C1 cells did not incorporate ^{35}S in the region identified as ASGP-1 (Figure 2B). Moreover, a second ^{3}H-labeled component (M_r 90–100 K) is not sulfated in either cell type. Following centrifugation of cells in CsCl–4 M Gdn·HCl, an ^{35}S peak was observed with the ^{3}H peak at a density of 1.4 g/cm^3 for MAT-B1 cells (data not shown), whereas no significant ^{35}S activity was observed for MAT-C1 cells at that gradient density.

We have isolated both MAT-B1 and MAT-C1 ASGP-1 by CsCl–4 M Gdn·HCl density gradient centrifugation of membrane fragments. The purified glycoprotein yields a single peak on electrophoresis in both polyacrylamide and 1% agarose electrophoresis gels and appears homogeneous by sedimentation velocity and gel filtration in 4 M Gdn·HCl (Sherblom et al., 1980). This isolation procedure was applied to double-labeled MAT-B1 cells. The density gradient profile of membrane fragments (Figure 3) shows that although the ^{3}H and ^{35}S labels band in the same region of the gradient, the peaks are not coincident. Throughout the isolation procedure, however, the ^{35}S/^{3}H ratio remains constant (Table I). A recovery of 15% of the total cell ^{3}H radioactivity in the purified glycoprotein is typical.

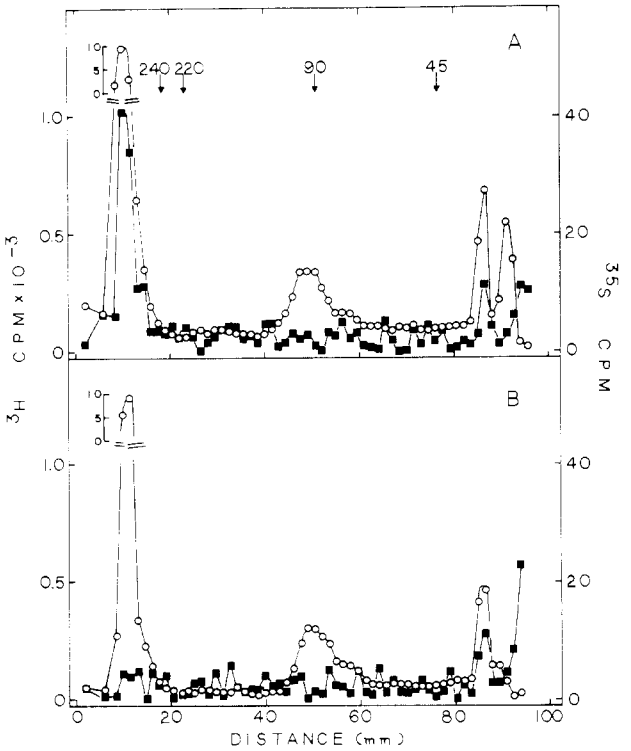

FIGURE 2: $NaDodSO_4$ gel electrophoresis of (A) MAT-B1 and (B) MAT-C1 cells labeled as in Figure 1. An aliquot of labeled cells was solubilized by addition of an equal volume of 3% $NaDodSO_4$, 0.125 M Tris, pH 6.8, and 5% β-mercaptoethanol and immersion in a boiling water bath. The sample was sonicated briefly, and glycerol and Pyronin Y were added prior to electrophoresis as described under Experimental Procedures. Fractions of 1.5 mL were solubilized, mixed with scintillation fluid, and counted to yield the radioactivity profiles for ^{3}H (○) and ^{35}S (■). Molecular weight markers were from a sample of bovine erythrocyte ghosts.

Table I: Recovery of ^{3}H and ^{35}S in Fractions from MAT-B1 Cells[a]

	% of cell radioact.		
	^{3}H	^{35}S	^{35}S/^{3}H
cells	100	100	0.14
glycosaminoglycan extract	44	46	0.14
membrane fragments	34	31	0.13
ASGP-1	15	16	0.16

[a] Conditions of labeling and isolation of fractions are described under Experimental Procedures. ^{3}H values were corrected for ^{35}S spillover.

Fractionation of Intact MAT-B1 ASGP-1. The density gradient profile (Figure 3) suggested that ASGP-1 might be heterogeneous with respect to sulfation since the ^{3}H and ^{35}S peaks were not coincident. Thus, the glycoprotein was subjected to two fractionation procedures in an attempt to separate sulfated from unsulfated molecules. First, isopycnic centrifugation in CsCl in the presence and absence of 4 M Gdn·HCl yielded an increase in ^{35}S/^{3}H with increasing density (data not shown). However, the shifts were not so dramatic as those observed in the original isolation (Figure 3) which combines both the rate zonal and isopycnic fractions. Secondly, the glycoprotein was chromatographed on DEAE-cellulose in the presence 6.0 M urea (data not shown). The glycoprotein eluted in a broad peak from 0.10 to 0.14 M NaCl, but the ^{3}H and ^{35}S profiles were coincident. Thus, there do not appear to be discrete sulfated and unsulfated populations of ASGP-1 which are separable on the basis of charge. The presence of

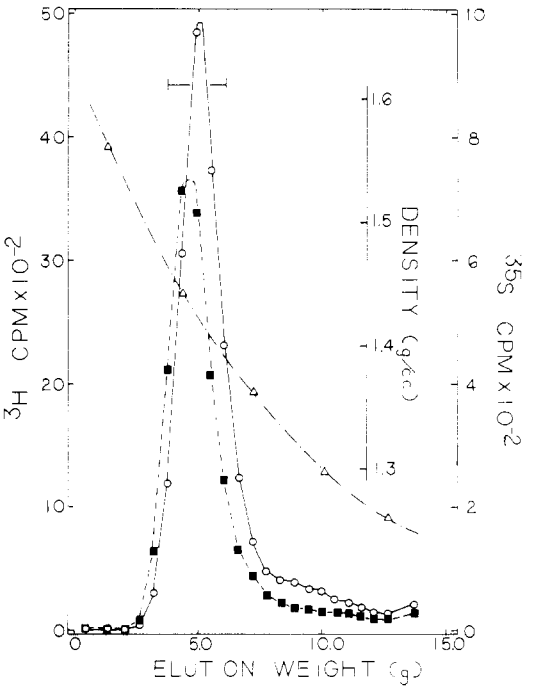

FIGURE 3: Purification of MAT-B1 ASGP-1 from membrane fragments in a CsCl gradient containing 4 M Gdn·HCl. Conditions of metabolic labeling, membrane isolation, and centrifugation are given under Experimental Procedures. Density gradient fractions were assayed for ^{3}H (○) and ^{35}S (■) radioactivity and for density (△). The bar denotes the fractions which were pooled to constitute ASGP-1.

sulfate, which can bind Cs^+ ions, greatly increases the effective density of a molecule, and the CsCl density gradient may be a more effective fractionation technique.

MAT-B1 ASGP-1 fractions from a CsCl–4 M Gdn·HCl gradient which differed in density and ^{35}S/^{3}H were analyzed in two ways. First, the gel filtration patterns (Bio-Gel P-4) following alkaline borohydride treatment were compared, and no obvious differences were noted in the ^{3}H or ^{35}S profiles. Second, carbohydrate analysis of the fractions revealed increases in the ratios of glucosamine, sialic acid, and fucose to galactosamine with increasing density (data not shown). More dramatic, however, was the carbohydrate/protein ratio expressed as nanomoles of GalNAc per microgram of protein. This ratio increased from 0.93 to 1.82 as the ^{35}S/^{3}H increased from 0.13 to 0.18. An increase in sulfation may be correlated with the degree of glycosylation of the protein. These results are consistent with the idea that sulfation is a relatively late biosynthetic event (Schachter, 1978).

Characterization of the Oligosaccharides from MAT-B1 ASGP-1. The oligosaccharides released by alkaline borohydride treatment of purified MAT-B1 ASGP-1 were fractionated by gel filtration followed by ion-exchange chromatography. We have previously reported compositions for the MAT-B1 oligosaccharides fractionated by gel filtration (Sherblom et al., 1980) which correspond to fractions I–V shown in Figure 4. In the present study, peaks III and IV were further divided into IIIa and IIIb and IVa and IVb since the ^{35}S and ^{3}H peaks were not coincident. Also, since the previous study indicated that peak I (void volume) contained primarily unreduced oligosaccharides, this sample was not used for further analysis.

Ion-exchange profiles for fractions II–V on DEAE-Sephadex are shown in Figure 5. One outstanding feature is that most of the ^{3}H resides in neutral oligosaccharides which do not bind to DEAE-Sephadex. Two major tritiated oligosaccharides which do bind to DEAE-Sephadex, II-4 and IIIa-2, do not

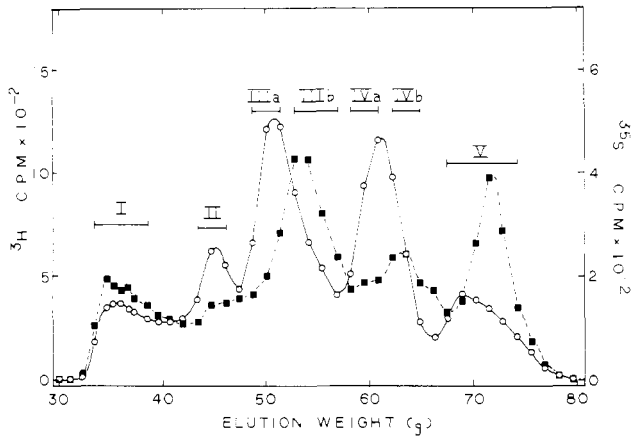

FIGURE 4: Gel filtration of alkaline borohydride treated MAT-B1 ASGP-1. Conditions of labeling and isolation of ASGP-1 are given under Experimental Procedures. ASGP-1 was incubated with 0.05 M NaOH and 1.0 M $NaBH_4$ for 16 h at 45 °C, neutralized with acetic acid, and applied to a 1.0 × 115 cm column of Bio-Gel P-4 equilibrated with 0.1 M pyridine acetate, pH 6.0. Fractions were assayed for ^{3}H (○) and ^{35}S (■) radioactivity and were pooled as indicated to constitute samples I–V.

Table II: Analysis of Oligosaccharides from MAT-B1 ASGP-1[a]

fraction	% galactosaminitol recovered	^{35}S/^{3}H	mol/mol of *N*-acetylgalactosaminitol			
			GlcNAc	Gal	SA	Fuc
II-1		0.05				
II-2	1.2	0.06				
II-4	3.2	0.03	1.21	2.16	4.04	0.12
II-5	0.7	0.65				
IIIa-1	0.2	0.08				
IIIa-2	11.7	0.04	1.21	1.93	2.04	0.25
IIIa-3	0.1	0.95				
IIIa-4	0.8	0.89				
IIIa-5	0.4	1.00				
IIIb-1	3.9	0.05	2.81	5.04	0.84	0.67
IIIb-2	4.9	0.01	1.15	2.83	2.61	0.24
IIIb-5	5.1	0.93	1.18	1.11	1.90	0.09
IVa-1	18.5	0.01	1.73	3.74	0.22	0.86
IVa-2	1.1	1.52	1.35	2.77	0.43	1.32
IVa-3	1.2	1.37	1.18	1.27	*b*	0.72
IVb-1	15.9	0.01	1.31	2.60	0.11	0.33
IVb-3	6.0	1.44	1.10	0.91	*b*	0.93
V-1	20.7	0.05	0.14	0.94	*b*	0.26
V-2	4.4	1.65	1.34	1.14	*b*	0.24

[a] Conditions for labeling, isolation of ASGP-1, alkaline borohydride treatment, and monosaccharide analysis are given under Experimental Procedures. Oligosaccharides were fractionated by gel filtration followed by ion-exchange chromatography. [b] None detected.

appear to be sulfated. From the labeling results, the proportion of oligosaccharides which is sulfated appears to be relatively small.

Compositional data for several of the oligosaccharides are given in Table II. From the recovery of galactosaminitol it is estimated that greater than 50% of the oligosaccharides are neutral and ~20% are sulfated. Since neutral oligosaccharides are not fractionated on DEAE-Sephadex, it is likely that the neutral fractions contain mixtures of oligosaccharides. Sulfated or sialylated fractions, on the other hand, are more likely to be homogeneous.

There are three major neutral fractions, one major sialylated oligosaccharide, and three major sulfated oligosaccharides. The neutral fractions V-1, IVb-1, and IVa-1 comprise 55% of the recovered galactosaminitol. The composition of V-1 suggests that it contains predominantly the disaccharide

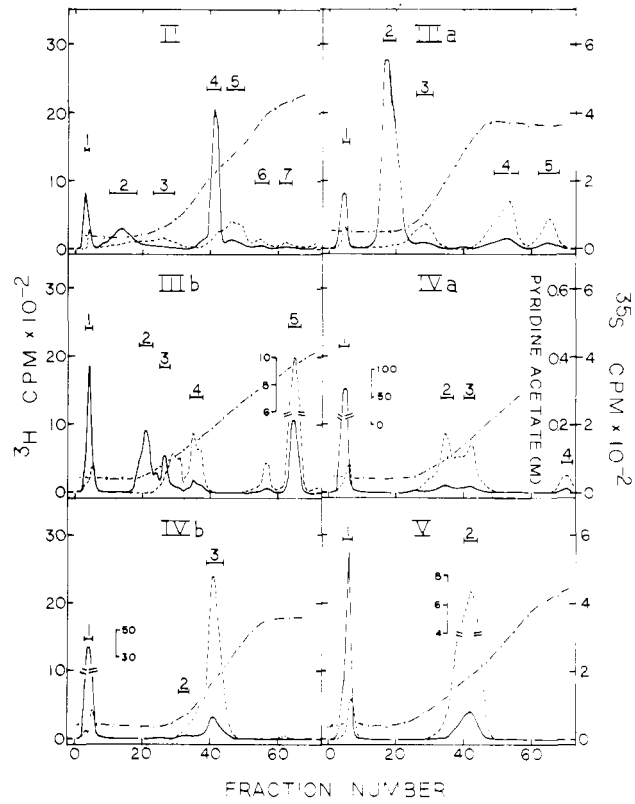

FIGURE 5: DEAE-Sephadex chromatography of oligosaccharides. Conditions of labeling, isolation of ASGP-1, alkaline borohydride treatment, and fractionation on Bio-Gel P-4 are described under Experimental Procedures. Oligosaccharide samples II–V were lyophilized, suspended in 0.05 M pyridine acetate, pH 5.0, and applied to a 1.5 × 20 cm column of DEAE-Sephadex A-25. The column was washed with the same buffer and eluted with a 240-mL linear gradient from 0.05 to 0.65 M pyridine acetate, pH 5.0. All graphs are plotted on the same scale, and all inset scales have units of cpm × 10^{-2}. Fractions were assayed for ^{3}H (—) and ^{35}S (- - -) radioactivity, and the conductance of selected fractions was measured to determine the pyridine acetate concentration (–·–). The bars denote fractions which were used for carbohydrate analysis.

Gal-GalNAcOH, although smaller amounts of fucosylated oligosaccharides may also be present. From gel filtration, the size of peak V is estimated to be 600, whereas the molecular weight of the disaccharide is 390. Fraction IVb-1 contains approximately 1 mol of GlcNAc and 2 mol of Gal per mol of galactosaminitol. The molecular weight of an oligosaccharide of this composition is 750, whereas the size of IVb is estimated to be 1000. Thin-layer chromatography of this fraction following acetylation yielded one tritiated band (data not shown). Fraction IVa-1, when acetylated and examined by thin-layer chromatography, separated into two components. Thus, the composition reflects the composition of a mixture of oligosaccharides with an average size of 1370, whereas the size of IVa estimated by gel filtration is 1200.

The major sialylated oligosaccharide is seen in fractions IIIa-2 and IIIb-2 which together comprise ~17% of the recovered galactosaminitol. These fractions apparently contain the same oligosaccharide based on the following: (1) each eluted from DEAE-Sephadex at approximately the same position (Figure 5); (2) their compositions were similar; (3) each fraction, when acetylated and examined by thin-layer chromatography, migrated to the same place and gave a single tritiated band. An oligosaccharide containing 1 mol of GlcNAc, 2 mol of Gal, and 2 mol of NANA per mol of galactosaminitol has a molecular weight of 1330, whereas the size of peak III is estimated to be 1800.

Three sulfated oligosaccharides together account for ~50% of the incorporated sulfate. Fraction V-2 represents ~4% of the total galactosaminitol. The composition is consistent with a trisaccharide of molecular weight 670 compared with an estimate of 600 by gel filtration. Fractions IVa-3 and IVb-3 comprise 7% of galactosaminitol and are apparently the same based on (1) similarity in $^{35}S/^{3}H$ ratio (1.37 vs. 1.44), (2) similarity in elution behavior from DEAE-Sephadex (0.16 vs. 0.17 M pyridine acetate), and (3) similarity in composition. The composition is consistent with a tetrasaccharide of 810 daltons compared with a gel filtration estimate of 1100. For the same reasons, fractions IIIa-5 and IIIb-5, which together comprise ~5% of the total galactosaminitol, are thought to be the same oligosaccharide. The size of this pentasaccharide is 1250 compared with an estimate of 1800 by gel filtration. These fractions appear to contain *both* sulfate and sialic acid on the same oligosaccharide, since a fraction of approximately the same size with the same number of sialic acid residues elutes from DEAE-Sephadex at a significantly lower pyridine acetate concentration (IIIa-2 and IIIb-2).

Discussion

MAT-B1 and MAT-C1 rat ascites adenocarcinoma cells differ substantially in several cell surface properties, including xenotransplantability. MAT-C1 cells will transplant into mice; MAT-B1 cells do not (Buck et al., 1979). Differences in ability to escape immune surveillance during transplantation have been related previously to the presence of a major cell surface sialoglycoprotein in the transplantable sublines that is absent in nontransplantable sublines (Sanford et al., 1973; Miller et al., 1977). However, the surface chemistry of both MAT-B1 and MAT-C1 sublines is dominated by the high molecular weight mucin-type glycoprotein ASGP-1 (Buck et al., 1979; Sherblom et al., 1980). Although the physical properties and amino acid compositions of MAT-B1 and MAT-C1 ASGP-1 are similar, there are significant differences in the oligosaccharides and in the content of sialic acid (Sherblom et al., 1980). It is yet uncertain if these changes affect xenotransplantability.

To determine if glycosaminoglycans or other glycoproteins might contribute to the differences in surface properties of the sublines, we metabolically labeled cells with [^{3}H]glucosamine and [^{35}S]SO_4^{2-}. There is a glycoprotein of molecular weight 90–100 K which incorporates 2 to 3% as much [^{3}H]glucosamine as the major glycoprotein ASGP-1. A small amount (<1%) of [^{3}H]glucosamine is incorporated into a sulfated glycosaminoglycan, which elutes from DEAE-cellulose at a concentration of 0.35 M NaCl. Although the labeling studies reveal no significant differences in these minor components between the two sublines, a striking difference is noted in the sulfate incorporation into ASGP-1. MAT-B1 cells incorporate sulfate into ASGP-1, although MAT-C1 cells do not. This is the most substantial difference found to date in the glycoprotein from the two sublines.

In the present work we have characterized the sulfated glycoprotein from the MAT-B1 ascites cells. Although sulfated glycoproteins have been described in several systems (Lombart & Winzler, 1974; Margolis & Margolis, 1970; Nemoto & Yosizawa, 1969; Endo & Yosizawa, 1975; Slomiany & Meyer, 1972; Nakamura & Compans, 1977), few structural studies have been performed (Lombart & Winzler, 1974; Slomiany & Meyer, 1972). Characterization of the glycoproteins may suggest biosynthetic differences between the two sublines which are important to differences in cell surface properties. We have previously characterized the MAT-B1 glycoprotein by composition and size (Sherblom et al., 1980). The present evidence pertains to the distribution of sulfate on the intact molecule and the oligosaccharides.

MAT-B1 ASGP-1 is isolated from a membrane fraction which is enriched in plasma membrane as determined by the activity of 5′-nucleotidase. Radioactivity incorporated from [^{3}H]glucosamine and [^{35}S]SO_4^{2-} coisolates during the fractionation procedure. There are not populations of sulfated and nonsulfated molecules which are separable on the basis of charge. When oligosaccharides released from double-labeled ASGP-1 by alkaline borohydride treatment are fractionated by sequential gel filtration and ion-exchange chromatography, sulfate is found to be distributed among several oligosaccharides. No consistent pattern for sulfation can be discerned, except that GlcNAc appears to be a required component. This is consistent with previous findings that GlcNAc is sulfated in mucin oligosaccharides (Lombart & Winzler, 1974). The compositional data suggest that the oligosaccharides have rather regular structures. Most of the data are consistent with a core structure containing GalNAc linked to repeating units of Gal and GlcNAc, which is variously substituted with fucose, sialic acid, and sulfate. However, in a few cases, notably II-4, IIIb-1, and IIIb-2, the number of Gal and/or sialic acid residues appears to be too great to be accommodated by this type of structure. These findings may result from variability in the accuracy of the carbohydrate analyses or from the presence of branching chains, probably containing Gal and/or sialic acid.

Whether sulfation contributes to differences in xenotransplantability and other cell surface properties is yet unknown. Also unknown is the mechanism by which the distribution of carbohydrates in the oligosaccharides of these glycoproteins is determined. Winzler (1973) has suggested a reciprocal relationship between sulfation and sialylation of mucin, determined by the transfer of sialic acid to GalNAc. However, our finding of both sialic acid and sulfate on one of the major sulfated oligosaccharides (IIIb-5) of MAT-B1 ASGP-1 suggests that this mechanism is not strictly applicable to this tumor cell system. It seems more likely that the mechanism(s) controlling these oligosaccharide structures is more complex. Further investigation of these oligosaccharides and the enzymes involved in their synthesis may aid in clarifying some of the questions concerning the control of cell surface properties by glycoproteins and the role of the cell surface in tumor cell survival.

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Variable Interaction of Spin-Labeled Human Myelin Basic Protein with Different Acidic Lipids†

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ABSTRACT: Myelin basic protein from the human central nervous system has been covalently spin-labeled with an iodoacetamide spin-label at its two methionyl residues at positions 21 and 167 from the N terminus of the protein. The spin-labeled protein, which is thought to bind electrostatically to acidic lipids followed by interaction of some hydrophobic segments with the lipid bilayer, was added to lipid vesicles in order to monitor the behavior of the protein in the lipid environment. A variety of lipids were used with which the degree of hydrophobic interaction seems to vary. On the basis of the effect of the protein on the organization of these lipids, the hydrophobic interaction seems to be greatest for phosphatidylglycerol and phosphatidic acid and decreases in the order phosphatidylserine > cerebroside sulfate ≥ phosphatidylethanolamine. The ESR spectra of the spin-labeled protein in lipid vesicles of phosphatidylglycerol and phosphatidic acid possessed two or three components at low temperatures, one immobilized and other more mobile components. At higher temperatures in these two lipids and in the other lipids at all temperatures, a single component mobile spectrum was observed with hyperfine splitting indicative of a relatively polar environment. However, the motional parameter τ_0 of the protein spin-label in vesicles was greater than that for the protein in solution and varied depending on the lipid. The greatest reduction of motion was observed with phosphatidylglycerol, followed in order by phosphatidic acid, cerebroside sulfate, phosphatidylserine, cardiolipin, and phosphatidylethanolamine. Measurement of the protein spin-label mobility at temperatures where the lipids possessed identical order parameters ($S = 0.4$) suggested that the order of the hydrocarbon chains of the different lipids was not the main factor in determining the probe motion. We conclude that the motion of the spin-label on the methionyl residues reflects different degrees of hydrophobic interaction of some regions of the protein with the bilayer and attribute this variability in hydrophobic interaction to the occurrence of intermolecular electrostatic and hydrogen bonding for some of the lipids.

In recent years, considerable work has been directed toward characterizing the interaction of the peripheral membrane proteins with different lipid species. Several types of interaction have so far been demonstrated (Papahadjopoulos et al., 1975; Susi et al., 1979). One type is characterized by the interaction of polylysine with lipids (Papahadjopoulos et al., 1975; Susi et al., 1979; Hartmann & Galla, 1978; Hammes & Schuller, 1970). In this case, the interaction involves an electrostatic binding of the polymer to the lipid head group with little or no hydrophobic interaction. A second type of interaction also involves electrostatic binding between protein and lipid, but the protein also appears to interact hydrophobically with the lipid bilayer, either by penetrating partway into the bilayer or by deforming the bilayer (Papahadjopoulos et al., 1975; Hartmann et al., 1978). There is considerable

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