THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 263, No. 18, Issue of June 25, pp. 8897-8903, 1988
Printed in U.S.A.

Sulfated *N*-Linked Oligosaccharides in Mammalian Cells

III. CHARACTERIZATION OF A PANCREATIC CARCINOMA CELL SURFACE GLYCOPROTEIN WITH *N*- AND *O*-SULFATE ESTERS ON ASPARAGINE-LINKED GLYCANS *

(Received for publication, October 21, 1987)

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In the preceding two papers, we described two new classes of sulfated *N*-linked oligosaccharides isolated from total cellular $^{35}SO_4$-labeled macromolecules of different mammalian cell lines. The first class carries various combinations of sialic acids and 6-*O*-sulfate esters on typical complex-type chains, while the second carries heparin and heparan-like sequences. In this study, we have characterized a sulfophosphoglycoprotein of 140 kDa from FG-Met-2 pancreatic cancer cells whose oligosaccharides share some properties of both these classes. The molecule was localized to the cell surface by electron microscopy using a monoclonal antibody (S3-53) and by cell surface ^{125}I-labeling. Metabolic labeling of the cells with radioactive glucosamine, methionine, inorganic sulfate, or phosphate all demonstrated a single 140-kDa molecule. Pulse-chase analysis and tunicamycin treatment indicated the glycosylation of a putative primary translation product of 110 kDa via an intermediate (120 kDa) to the mature form (140 kDa). Digestion with peptide:*N*-glycosidase F (PNGaseF) indicated a minimum of four *N*-linked glycosylation sites. PNGaseF released more than 90% of the [6-^{3}H]$GlcNH_2$ label and 40–70% of $^{35}SO_4$ label from the immunoprecipitated 140-kDa molecule. The isolated oligosaccharides were characterized as described in the preceding two papers. The majority of [6-^{3}H]$GlcNH_2$-labeled molecules were susceptible to neuraminidase. More than 50% of the $^{35}SO_4$ label was associated with only 5–10% of the ^{3}H-labeled chains. Some of the sulfated chains were partly sialylated molecules with four to five negative charges. Treatment with nitrous acid released about 25% of the $^{35}SO_4$ label as free sulfate, together with 6% of the [6-^{3}H]$GlcNH_2$ label, indicating the presence of *N*-sulfated glucosamine residues. Some of these oligosaccharides were degraded by heparinase and heparitinase. Therefore, while they are not as highly charged as typical heparin or heparan chains, they must share structural features that permit recognition by the enzymes. Thus, this 140-kDa glycoprotein contains at least four asparagine-linked chains substituted with a heterogeneous mixture of sulfated sequences. The heterogeneity of these molecules is as extensive as that described for whole-cell sulfated *N*-linked oligosaccharides in the preceding two papers.

The tissue distribution of six antigens recognized by different monoclonal antibodies to the human pancreatic cancer cell line COLO 357 and its subclones has recently been reported (1). One of these, a glycoprotein of 140 kDa, is present in cells of epithelial, mesodermal, and neuroectodermal origin and is widely distributed among human cell lines and tissues. However, the molecule seems specific for acinar cells of the normal adult pancreas, whereas in the fetal organ and in pancreatic carcinomas it is present on both acinar and ductal cells (1). We describe here several biochemical properties of this glycoprotein, in particular the carbohydrate moiety which carries some unusual post-translational modifications.

In the preceding two papers (2, 3) we described in detail two new classes of sulfated *N*-linked oligosaccharides in mammalian cells. We show here that the *N*-linked sugar chains of this 140-kDa antigen include sulfated molecules with properties intermediate between these two classes.

EXPERIMENTAL PROCEDURES

Many of the materials and methods used in this report are described in detail in the two preceding papers (2, 3).

Antibodies—Monoclonal antibody S3-53 recognizes an antigen in the human pancreatic carcinoma cell line COLO 357 and its subclones FG, SG, and FG-Met-2 (1). Monoclonal antibody Q5/13 directed against the β chains of most human Ia molecules (HLA-DR and DQ) was used as a negative control for immunoprecipitation (4), since the above cell lines lack such surface molecules.

Cell Culture—The metastatic subclone FG-Met-2 used in this study was cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 50 μg/ml gentamycin.

Electron Microscopy—This was performed essentially as described (5). Briefly, colloidal gold particles were conjugated with rabbit antimouse IgG and sized by ultracentrifugation. Cells were grown on 35-mm Petri dishes, fixed (2% paraformaldehyde, 0.75 M lysine) for 30 min, and incubated with monoclonal antibody S3-53 (100 μg/ml) for 4 h at room temperature in 5% bovine serum followed by gold-labeled rabbit antimouse IgG for 4 h. Cells were refixed and prepared for transmission electron microscopy.

Cell Labeling—The conditions used for intrinsic or cell surface labeling of FG-Met-2 cells varied with the radioactive isotope. Exponentially growing cell monolayers at about 80% confluency were radiolabeled for various lengths of time in different media as follows: [6-^{3}H]$GlcNH_2$ (40 Ci/mmol, Amersham Corp.), 1 mCi/ml for 48–72 h in RPMI 1640 medium with 5% fetal calf serum; [^{35}S]methionine (1295 Ci/mmol, Du Pont-New England Nuclear); 100 μCi/ml for 12 h in methionine-free RPMI 1640 medium (Flow Laboratories) containing 3% dialyzed fetal calf serum, 2 mM glutamine, and 50 μg/ml gentamycin; [^{35}S]sulfuric acid (40 Ci/mg of sulfur), 1 mCi/ml for 6 h

* This research was supported by Grants CA38701 and CA32925 from the United States Public Health Service. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Recipient of an Established Investigatorship of the American Heart Association.

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in sulfate label incorporation medium (see Ref. 2); or [^{32}P]phosphoric acid (285 Ci/mg of phosphorus, Du Pont-New England Nuclear), 0.5 mCi/ml for 3 h in phosphate-free medium (Irvine Scientific). Cell surface labeling with [^{125}I]sodium iodide (350–600 mCi/mmol, Amersham Corp.) was carried out with 2 mCi/ml for 15 min at room temperature using Enzymobead radioiodination reagent (Bio-Rad). The labeled cells (except for the ^{32}P label) were detached using phosphate-buffered saline, pH 7.5, containing 10 mM EDTA and 0.02% KCl, washed twice with cold 10 mM Tris-HCl, pH 7.5, and lysed on ice for 30 min in 10 mM Tris-HCl, pH 7.5, containing 2% Renex 30, 1 mM $CaCl_2$, 0.02% NaN_3, and freshly added 2 mM phenylmethylsulfonyl fluoride (lysis buffer). After ^{32}P labeling, cells were washed twice with cold phosphate-buffered saline containing 100 mM NaP_i; 100 mM NaF, 4 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and lysed for 10 min on ice in lysis buffer with these additions. The cell lysates were cleared by ultracentrifugation at 100,000 × *g* for 60 min at −4 °C and stored at −70 °C with analysis.

Pulse-Chase Analysis—Single cell suspensions of exponentially growing FG-Met-2 cells were propagated for 1 h at 37 °C in methionine-free medium (Irvine Scientific) and pulsed for 10 min with [^{35}S]methionine (1 mCi/3 × 10^7 cells/ml). An aliquot of cells representing the zero time point was removed, and the remaining cells were washed three times with cold 10 mM Tris-HCl, pH 7.5, containing 10 mM unlabeled methionine, resuspended in GM 1717 medium (with 10 mM methionine), and incubated on a rocker platform at 37 °C. Aliquots were removed at different times and processed as described above.

Tunicamycin Treatment—Semiconfluent monolayers were incubated for 12 h in the presence of 1 μg/ml tunicamycin and pulsed with [^{35}S]methionine for 12 h in methionine-free RPMI containing 3% dialyzed fetal calf serum and 1 μg/ml tunicamycin. Control flasks, similarly labeled with [^{35}S]methionine in the absence of tunicamycin, were also prepared. This tunicamycin concentration appeared optimal since complete inhibition of *N*-linked glycosylation was achieved while the inhibition of protein synthesis was minimal (<15%), as measured by trichloroacetic acid precipitation of the ^{35}S-labeled lysates.

Immunoprecipitation—Intrinsically labeled cell lysate (1–10 × 10^6 cpm) in 300 μl of 10 mM Tris-HCl, pH 8.5, 150 mM NaCl, 0.5% Tween 20, 0.1% Renex 30, 2.5 mM NaN_3, 0.1 mg/ml ovalbumin was immunoprecipitated with 10 μl of a 10% suspension of monoclonal antibody conjugated to CNBr-activated Sepharose CL-4B (Pharmacia LKB Biotechnology Inc.) by overnight incubation at 4 °C. The immunoadsorbents were washed 8 times with the same buffer and 2 times with 10 mM Tris-HCl, pH 8.5, 150 mM NaCl, 0.5% Tween 20, 2.5 mM NaN_3. Bound antigens were eluted by boiling in Laemmli sample buffer (6) for polyacrylamide gel electrophoresis with 0.5% SDS[1] containing 50 mM 2-mercaptoethanol for PNGaseF digestions or with 50 mM diethylamine, pH 11, containing 1% octyl glucoside for trypsin digestions. The latter eluate was immediately neutralized with Tris-HCl and dialyzed against the 50 mM ammonium bicarbonate containing 0.1% Renex 30. If necessary, 0.2% SDS was included in the washing buffers to minimize nonspecific background.

Polyacrylamide Gel Electrophoresis—Samples were analyzed by SDS-PAGE according to Laemmli (6) and visualized by autoradiography. Molecular weight standards were from Bio-Rad: myosin (200 kDa); β-galactosidase (116 kDa); phosphorylase *b* (92 kDa); bovine serum albumin (68 kDa); and ovalbumin (45 kDa). Two-dimensional gel analysis was performed as described (7). Briefly, immunoprecipitates were eluted with 9.5 M urea for 30 min at room temperature and subjected to nonequilibrium electrophoresis in tube gels in the first dimension (Ampholines, pH 3.5–10), followed by 7.5% SDS-polyacrylamide gels in the second dimension.

RESULTS

Identification of the 140-kDa Glycoprotein—Fig. 1 shows an autoradiograph obtained by SDS-PAGE analysis of immunoprecipitates from [^{35}S]methionine-labeled FG-Met-2 cells. One band of M_r 140,000 was observed under reducing conditions (Fig. 1, *lane a*) which migrated as M_r 130,000 without reduction (Fig. 1, *lane b*). No bands were seen using anti-HLA antibodies Q5/13 for immunoprecipitation (Fig. 1, *lane c*). This molecule was also intrinsically labeled with other radioisotopes and subjected to one- and two-dimensional electrophoresis. As can be seen in Fig. 2, *a–e*, the 140-kDa molecule was immunoprecipitated from cells intrinsically labeled with either methionine, glucosamine, inorganic sulfate, or phosphate or surface-labeled with iodine. Using [^{35}S]methionine-labeled cells a single spot was observed on two-dimensional analyses (Fig. 2*f*).

Cell Surface Localization—Since labeling with ^{125}I was performed under conditions which only label the cell surface and

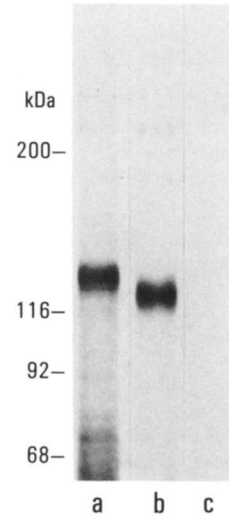

FIG. 1. **Identification of the 140-kDa glycoprotein by SDS-PAGE analysis using mAb S3-53 for immunoprecipitation.** The human pancreatic cancer cell line FG-Met-2 was intrinsically labeled with [^{35}S]methionine and lysed in buffer containing 2% Renex (for details see under "Experimental Procedures"). The lysate was immunoprecipitated with mAb S3-53 and analyzed by SDS-PAGE on a 5% gel under reducing (*a*) or nonreducing conditions (*b*). No bands were seen (*c*) when mAb Q5/13 directed against the β-chain of HLA-DR and DQ was used for immunoprecipitation. Autoradiographs were exposed to film for 3 days.

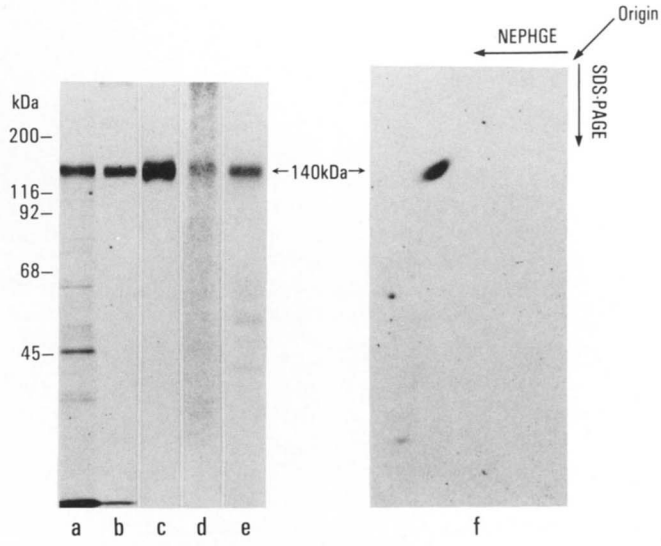

FIG. 2. **Immunoprecipitation of cell lysates from FG-Met cells labeled with different radioisotopes as displayed by one- or two-dimensional polyacrylamide gels.** Cells were intrinsically labeled with: *a* and *f*, [^{35}S]methionine; *b*, [^{35}S]sulfate; *c*, [6-^{3}H]glucosamine; *d*, [^{32}P]phosphate and cell surface labeled with *e*, [^{125}I]iodine. Cell lysates were immunoprecipitated with mAb S3-53 and run on 10% polyacrylamide gels under reducing conditions (*a–e*) or by two-dimensional gel electrophoresis (*f*). Autoradiographs were exposed to film for 3–5 days (for details see under "Experimental Procedures").

[1] The abbreviations used are: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PNGaseF, peptide:*N*-glycosidase F; mAb, monoclonal antibody.

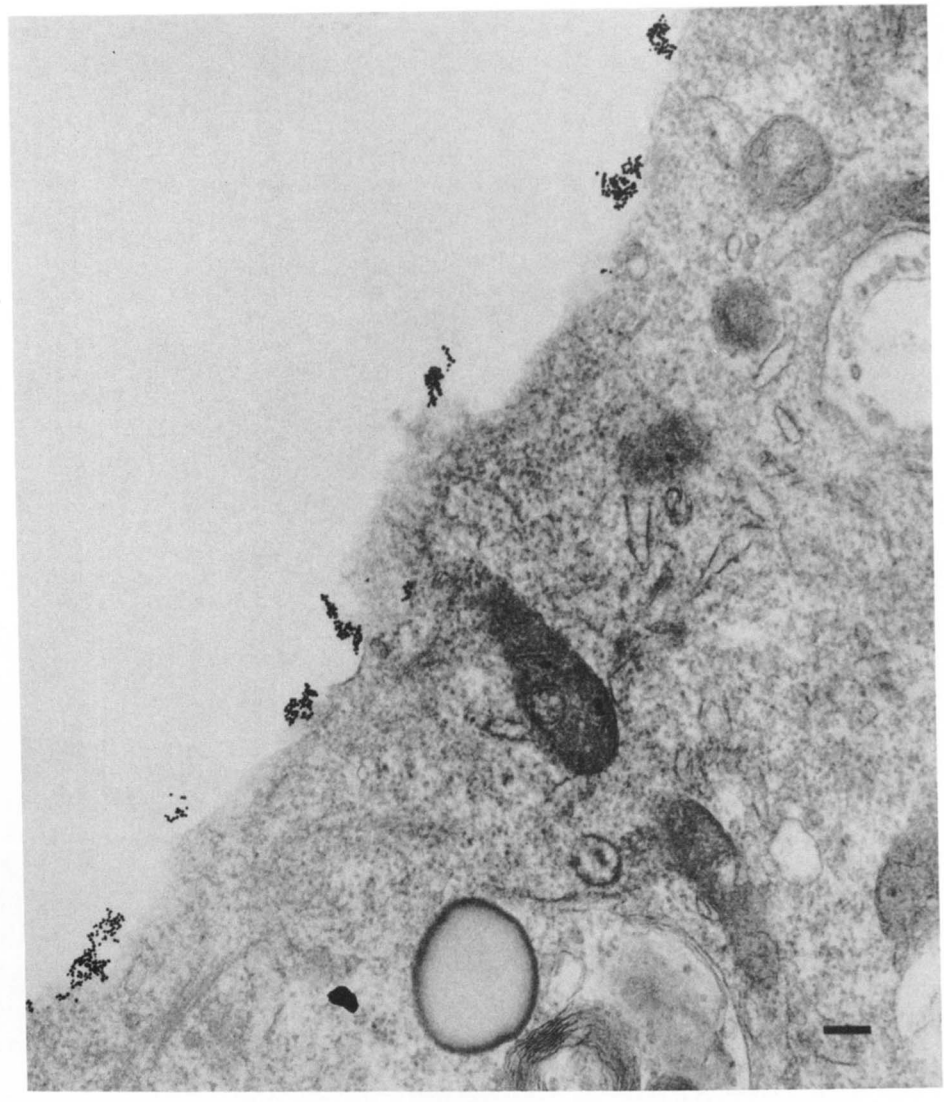

FIG. 3. **Localization of the 140-kDa glycoprotein on FG-Met-2 cell surfaces by electron microscopy.** Fixed FG-Met-2 cells were incubated with mAb S3-53 followed by rabbit antimouse IgG conjugated to gold particles (3–5 nm). *Bar* = 0.2 μm. No staining was observed when myeloma supernatant P3X63 Ag8 was used. The *bar* represents 1 μM (for details see under "Experimental Procedures").

immunofluorescence studies showed cell surface staining (1), electron microscopy studies were undertaken to further localize this glycoprotein. Fig. 3 shows an electron micrograph obtained by incubating FG-Met-2 cells with mAb S3-53 followed by rabbit antimouse IgG conjugated to gold particles. Clusters of gold particles were observed on the outer cell surface and/or extracellular matrix regions. This distribution is similar to that observed with other surface proteins such as human placental alkaline phosphatase on placental and cancer cells (5). No staining was seen when myeloma supernatant was used.

Biosynthesis—As shown in Fig. 4, pulse-chase analyses of [^{35}S]methionine-labeled cells indicated that a 120-kDa molecule is converted to the mature 140-kDa product after 1 h and that this molecule has a half-life of less than 10 h. The 140-kDa molecule is not degraded by endo-β-*N*-acetylglucosaminidase H, indicating a lack of high mannose chains, but is degraded by PNGaseF to M_r 110,000. Treatment of the cells with tunicamycin, an inhibitor of *N*-linked glycosylation, also indicated the presence of a 110-kDa primary translation product (Fig. 4). The migration of the molecule is slightly affected by neuraminidase (less than 10-kDa shift on polyacrylamide gels; data not shown) indicating that sialic acid residues are present. Thus, it appears that as with many other glycoproteins, this molecule is co-translationally *N*-glycosylated and and the oligosaccharides are then processed to complex-type chains. The finding with tunicamycin also indicates that mAb S3-53 recognizes a protein epitope rather than an oligosaccharide structure.

Number of N-Linked Glycosylation Sites—To obtain an estimate of the number of *N*-linked glycosylation sites, the immunoprecipitated 140-kDa glycoprotein from [^{35}S]methionine-labeled cells was treated with increasing amounts of PNGaseF and subjected to SDS-PAGE analyses. As can be seen in Fig. 5, a "ladder" pattern was obtained, with each rung representing the release of successive *N*-linked oligosaccharide chains. Although the reproduction of the fluorograph is technically poor, the original allows a minimum of four such chains to be distinguished.

Release of Sulfated N-Linked Oligosaccharides by PNGaseF—The isolated 140-kDa glycoprotein from $^{35}SO_4$-labeled FG-Met-2 cells was treated with or without PNGaseF and the radioactivity fractionated on a Sephacryl S-200 column (Fig. 6). The control sample incubated without enzyme eluted as a symmetrical peak in the void volume (*left panel*). Digestion with PNGaseF released about 70% of the sulfate label indicating that the majority of ^{35}S label was present in *N*-linked oligosaccharides. Redigestion of the excluded material with PNGaseF gave no further release, suggesting that the remaining sulfate might be bound to the peptide and/or to *O*-linked

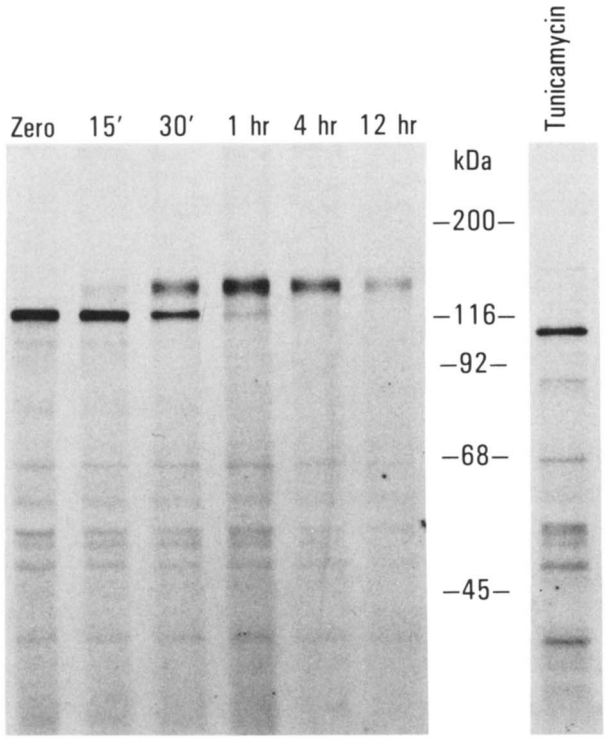

FIG. 4. **Pulse-chase analysis and tunicamycin treatment of FG-Met-2 cells.** FG-Met-2 cells were pulsed with [^{35}S]methionine for 10 min and chased for the times indicated (*left panel*). The cell lysates were immunoprecipitated with mAb S3-53 and analyzed on a 10% polyacrylamide gel in SDS under reducing conditions. Cells were also incubated with 1 μg/ml of tunicamycin, intrinsically labeled with [^{35}S]methionine and analyzed as above (*right panel*).

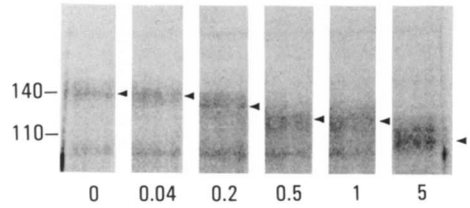

FIG. 5. **Minimum number of *N*-linked glycosylation sites detected by PNGaseF digestion.** Cell lysate from [^{35}S]methionine-labeled cells was immunoprecipitated with mAb S3-53 and the isolated 140-kDa molecule treated with increasing concentrations of PNGaseF as indicated for 1 h. The samples were analyzed on 10% polyacryamide gels in SDS and studied by fluorography. Each *arrow* indicates a distinct stepwise decrease in apparent molecular weight.

oligosaccharides. Fig. 7 shows similar results obtained using cells intrinsically labeled with both $^{35}SO_4$ and [6-^{3}H]GlcNH$_2$. More than 90% of the ^{3}H label and about 40% of the $^{35}SO_4$ radioactivity was released by PNGaseF digestion. Fractions were pooled as shown to give two classes of released *N*-linked oligosaccharides: R1, containing all of the released $^{35}SO_4$ label and 50% of the glucosamine label, and R2 representing the other 50% of the released glucosamine label.

QAE-Sephadex Analysis of N-Linked Oligosaccharides—Batchwise elution from QAE-Sephadex has previously been used to obtain an estimate of the extent of negative charge on labeled oligosaccharides (8). Fractions R1 and R2 were studied in this manner with or without prior neuraminidase treatment. As shown in Fig. 8 the R2 fraction contains anionic oligosaccharides with 0, 1, and 2 negative charges (25, 40, and 30%, respectively). After enzyme treatment, 65% of the ^{3}H radioactivity was rendered neutral, and 20% of the label now eluted at the position expected for free sialic acid (20 mM

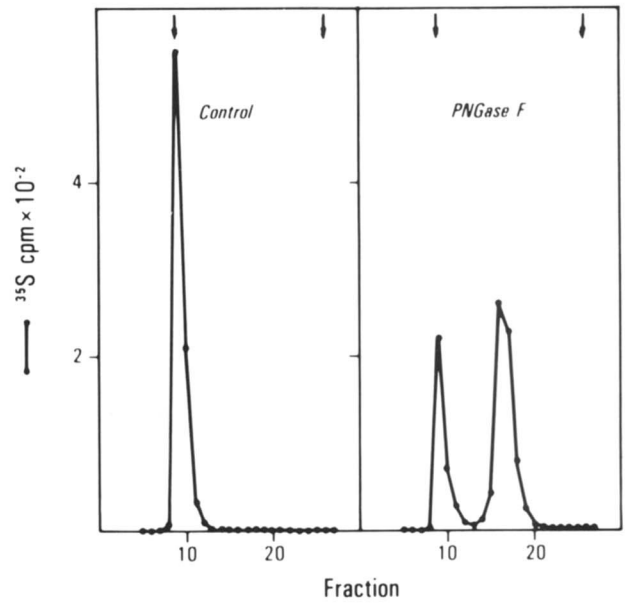

FIG. 6. **Release of sulfated *N*-linked oligosaccharides from the $^{35}SO_4$-labeled 140-kDa molecule.** The 140-kDa glycoprotein was isolated from [^{35}S]sulfate-labeled FG-Met-2 cells by immunoprecipitation and incubated without (*left panel*) or with (*right panel*) PNGaseF. The reactions were analyzed for release of sulfated oligosaccharides by Sephacryl S-200 chromatography (see Ref. 2 for details).

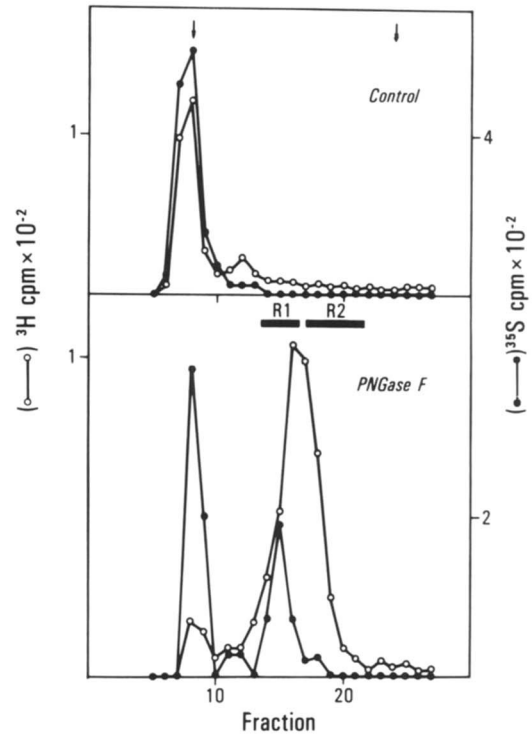

FIG. 7. **Release of *N*-linked oligosaccharides from the [^{35}S] sulfate and [^{3}H]glucosamine-labeled 140-kDa molecule by PNGaseF.** The 140-kDa glycoprotein was isolated from FG-Met-2 cells intrinsically labeled with [^{35}S]sulfate and [^{3}H]glucosamine, digested with PNGaseF, and separated on a Sephacryl S-200 column in 10 mM Tris-HCl, pH 6.5, containing 0.2% SDS. Blue Dextran and [^{3}H]Man were used as markers for V_0 and V_{100}, respectively.

NaCl). This is not unexpected, since [^{3}H]GlcNH$_2$ can label sialic acid. Thus, it appears that most if not all of the negative charge in R2 oligosaccharides is due to sialic acid residues. The QAE-Sephadex elution profile of R1 which contains both

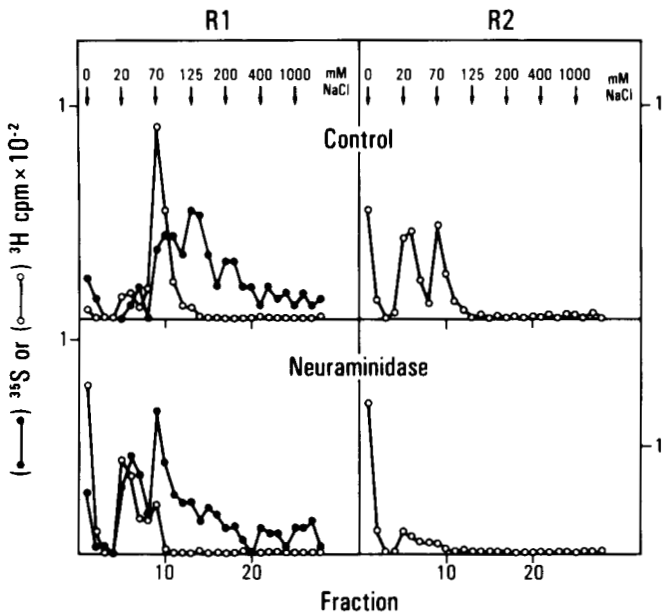

FIG. 8. **QAE-Sephadex anion exchange chromatography of *N*-linked ^{3}H,^{35}S-labeled oligosaccharides released by PNGaseF.** The isolated oligosaccharide fractions R1 and R2 were desalted, applied to QAE-Sephadex columns before and after neuraminidase treatment, and step eluted with the indicated concentrations of NaCl in 2 mM Tris base (see Ref. 2 for details).

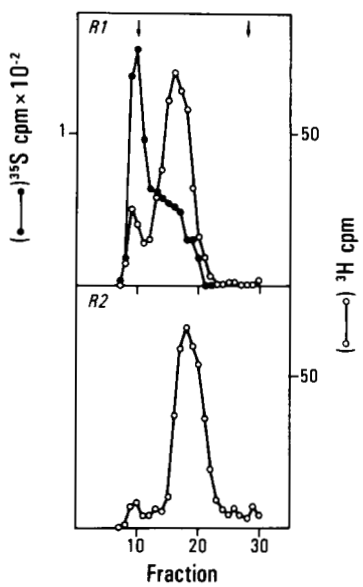

FIG. 9. **Sephadex G-50 analyses of PNGase-F released *N*-linked oligosaccharides.** PNGaseF-released oligosaccharides R1 and R2 were separated on Sephadex G-50 columns in 10 mM Tris-HCl, pH 6.5, containing 0.2 SDS. Blue dextran and [^{3}H]Man were used as markers (*arrows*) for the V_0 and V_{100}, respectively.

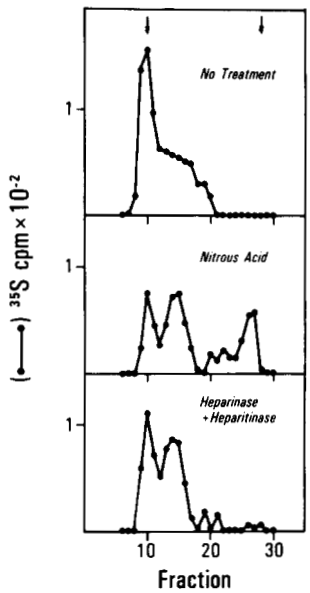

FIG. 10. **Sephadex G-50 analyses of PNGaseF-released sulfated *N*-linked oligosaccharides treated with nitrous acid and heparinase/heparitinase.** PNGaseF-release oligosaccharides from fraction R1 were treated with nitrous acid or heparinase/heparitinase and fractionated on Sephadex G-50. For clarity only the ^{35}S label is shown; the corresponding ^{3}H profiles are discussed in the text. Blue Dextran and [^{3}H]Man were used as markers in each run to identify the V_0 and V_{100}, respectively. See Ref. 3 and under "Experimental Procedures" for details.

labels was more complex (see Fig. 8). Several conclusions can be made from this experiment. First, about 60% of the sulfate label was associated with less than 5% of the ^{3}H label in molecules with higher negative charge (requiring 125 mM NaCl or higher for elution). Second, 75% of the ^{3}H label eluted at the position of 2 negative charges (70 mM NaCl). Upon neuraminidase treatment, most of this ^{3}H radioactivity shifted to molecules that were neutral or had one negative charge. Third, about half of the sulfate label eluting with 125 and 200 mM NaCl shifted to the elution positions for 1 and 2 negative charges when digested with neuraminidase. Thus, some of the sulfate label is on chains that also carry sialic acids.

Only a Minor Fraction of the N-Linked Oligosaccharides Is Sulfated—The preceding data suggest that while most of the oligosaccharides carry sialic acid residues, a minority carry either sulfate esters alone or combinations of sulfate esters and sialic acid. Further evidence for this was obtained by exclusion chromatography on Sephadex G-50. As shown in the *upper panel* of Fig. 9 about 50% of the sulfate label from R1 appeared in the void volume together with 10% of the tritium radioactivity, again demonstrating that a high proportion of the sulfate is associated with a minor part of the total oligosaccharides. The remaining sulfate and tritium radioactivity eluted together, at a position similar to that of R2 oligosaccharides, containing only ^{3}H label (*lower panel* of Fig. 9).

The Asparagine-linked Oligosaccharides Contain N-Sulfate Esters—To further characterize the sulfated oligosaccharides, R1 was treated with nitrous acid or combined heparinase/heparitinase and analyzed on Sephadex G-50. For clarity and easy comparison only the $^{35}SO_4$ elution profiles are shown in Fig. 10, and the *upper panel* is the same as the *upper panel* of Fig. 9. Nitrous acid deamination at pH 1.5 (9) released about one-fourth of the total sulfate label in R1, which is accounted for by a loss of about half of the radioactivity originally excluded from the column. The nitrous acid-released radioactivity now appeared at the position expected for free sulfate. Similarly, 6% of the ^{3}H label shifted from the void volume and co-eluted with the sulfate label (data not shown). This indicates that a significant proportion of the total $^{35}SO_4$ label in R1 is present as *N*-sulfated glucosamine. *N*-Sulfate esters have been previously described almost exclusively in heparin/heparan sulfate glycans. The sulfated oligosaccharides studied here showed a much lower charge density than that seen for typical heparan or heparin sulfate glycosaminoglycans. However, when an aliquot of R1 was incubated with enzymes that

degrade such structures, a shift of the $^{35}SO_4$ label was obtained, with about 25% of excluded radioactivity shifting into the included volume (*lower panel* of Fig. 10). This finding indicates that about half of the chains carrying *N*-sulfate groups (probably on glucosamine) are sensitive to heparinase/heparitinase. These enzymes are known to have very definite specificity and do not even degrade all forms of heparin/heparan sulfate (10). Thus, these oligosaccharides must have very short heparin/heparan sulfate-like sequences attached to the *N*-linked oligosaccharide core.

DISCUSSION

In the two preceding papers (2, 3) we studied total cellular sulfated *N*-linked oligosaccharides from various mammalian cell lines and described two general classes of these molecules. The first class was complex-type chains carrying sialic acids and *O*-sulfate esters, while the second was molecules carrying heparin/heparan-like chains. In contrast, the present report describes a single antigen defined by a monoclonal antibody. This 140-kDa glycoprotein expressed by a human pancreatic carcinoma cell line was studied with respect to general properties such as cellular localization and biosynthesis but with special emphasis on its carbohydrate moieties. The protein was located at the cell surface as judged by electron microscopy and immunoprecipitation of cell surface iodinated molecules. The results of pulse-chase analysis and tunicamycin treatment show a pattern of biosynthesis of *N*-linked oligosaccharides that is similar to that seen in many other glycoproteins. Thus, the mature 140-kDa molecule is likely to result from co-translational glycosylation of a 110-kDa peptide with dolichol-P-P-oligosaccharide to a size of 120 kDa; further processing of the *N*-linked chains to complex-type oligosaccharides results in the subsequent increase in molecular weight. We do not know whether *O*-linked oligosaccharides are present on the protein; if they are, they do not contribute in a major way to the apparent molecular weight.

The glycoprotein is also post-translationally modified by sulfation and phosphorylation. The majority of the sulfate esters was shown to be on *N*-linked oligosaccharides by specific release with PNGaseF. The evidence against artifacts that could provide alternate explanations for this release have been discussed in detail in the two preceding papers (2, 3). This discussion will not be repeated here, except to state that each of the possible artifacts can be ruled out. Nitrous acid treatment of the sulfated oligosaccharides showed that both *N*- and *O*-sulfate esters were present. The labeled chains were partially susceptible to both neuraminidase and to heparin/heparan degrading enzymes. Thus, the sulfated chains on this protein appear to share properties of both classes of oligosaccharides described in the two preceding papers (2, 3). On the one hand, most of the chains are relatively low in charge density and have sialic acid residues (properties of Class I oligosaccharides). On the other hand, they carry *N*-sulfate esters and have heparin/heparan-like sequences (properties of Class II molecules). Sizing by Sephadex G-50 chromatography also shows some overlap between the two classes. Thus, the heterogeneity found in total cellular sulfated *N*-linked oligosaccharides is mirrored in the chains found on a single protein.

The presence of *N*-glycosidically linked nitrous acid-sensitive sulfate esters in calf thyroid plasma membrane glycoproteins has been previously reported (11). In contrast, *O*-sulfate esters have been reported on a variety of glycoproteins (12–15). To our knowledge, this is the first description of *N*-sulfate esters on asparagine-linked oligosaccharides of a specific protein.

The changes in molecular weight upon tunicamycin and PNGaseF treatment suggest the presence of four to six complex-type *N*-linked chains. Treatment with varying amounts of PNGaseF indicates a minimum of four glycosylation sites. In spite of this, >50% of the sulfate label was found associated with less than 10% of the glucosamine label. One explanation could be that a minority of the protein molecules carries the sulfated oligosaccharides. The other possibility is that only a minority of the oligosaccharide chains is sulfated. Our preliminary experiments in this regard indicate a very complex situation, with the extent and distribution of sulfation being variable, apparently dependent upon the phase of cell growth and/or the culture conditions.[2] This variability does not appear to be random and could be related to the biological role of sulfation. We are currently studying this matter in greater detail.

The biological significance of sulfate esters on *N*-linked oligosaccharides is largely unknown. The relative rarity of these modifications and the limited extent of modification on even a single protein make their frequency similar to that of the mannose 6-phosphate residues of lysosomal enzymes (8) and suggest that they could have equally important biological roles. On the other hand, increasing evidence shows that the sulfated proteoglycans in general and heparan sulfate, in particular, play important physiological roles (16–19). Different structures comprising about 5–10 sugar residues of heparin, heparan, and dermatan sulfate chains are involved in defined molecular interactions that can mediate specific biological functions (20–23). We have shown here that there can be considerable overlap between the structures of sulfated glycoproteins and proteoglycans. This remarkable heterogeneity arising from extensive post-translational modifications increases the range of possibilities for such specific structure-function relationships.

Acknowledgments—We would like to thank George Klier for performing the electron microscopy studies and Linda Roux for stimulating discussions.

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