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Glycoproteins in Culture Medium: A Comparison from Cystic Fibrosis and Control Skin Fibroblasts†

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ABSTRACT: High molecular weight glycoproteins have been partially purified by four methods from the medium after growth of human skin fibroblasts. One of these methods, precipitation by heparin, yields one major glycoprotein, M_r 220000, when examined by polyacrylamide gel electrophoresis under denaturing conditions. The glycoprotein was labeled with L-[^{3}H]fucose or D-[^{3}H]glucosamine and yielded glycopeptides after digestion with Pronase. Using this method of precipitation, we made a comparison of matched cystic fibrosis (CF) and control human skin fibroblast media with and without 10% fetal calf serum, a nutritive requirement for growth. The profiles of the [^{3}H]fucose-labeled material from media of both cell types were similar after polyacrylamide gel electrophoresis regardless of the procedure used to obtain them. In contrast, differences were seen in the composition of the material obtained by precipitation with heparin, which suggested differences in glycosylation by CF fibroblasts. In addition, differences were noted which were related to the presence or absence of fetal calf serum in the growth medium. The analysis showed that when the CF and control materials were compared, the heparin precipitate of the CF medium without fetal calf serum contained (1) less protein and carbohydrate, (2) 30–35% less radioactivity per milligram of protein, (3) a lowered fucose content, (4) higher sialic acid content in confluency, and (5) lower turnover when expressed as counts per minute per nanomole of fucose. When the medium from the CF fibroblasts contained fetal calf serum, the heparin-precipitated material had more radioactivity and a higher ratio of fucose to other monosaccharides than that from control cells. Thus, there was either a differential response of the CF and control fibroblasts grown in the presence of 10% fetal calf serum or a differential recruitment of other glycoproteins during the precipitation of the serum-containing medium. The results are discussed in relation to previous findings of the monosaccharide content of CF fibroblast membranes and CF secretions.

Glycoproteins of high molecular weight are found associated with the cell surface or in the growth medium of fibroblasts and other cell types (Glick & Flowers, 1978). The relationships of these glycoproteins, for example, fibronectin, to biological functions such as cell adhesion, morphology, transformation, and embryogenesis have been reviewed (Vaheri & Mosher, 1978; Frazier & Glaser, 1979; Kobata, 1979; Yamada et al., 1980). The complexity of the interactions of high molecular weight glycoproteins with glycosaminoglycans and other macromolecules has been reported (Ruoslahti et al., 1980). Another glycoprotein, Excitoporin, was reported to be associated with neurites and membranes of differentiating mouse (Littauer et al., 1980) or human (Littauer et al., 1979) neuroblastoma cells in culture. When the cells were not differentiated, a glycoprotein of similar size subunits was found in the culture medium. Whether or not the glycoproteins in the culture medium represent material shed from the membrane and/or internal products secreted from the cells is not fully understood. The difficulty in purifying these glycoproteins for characterization has impaired progress in this area (Glick & Flowers, 1978; Kobata, 1979; Momoi et al., 1980).

Membrane glycoproteins from skin fibroblasts of patients with cystic fibrosis (CF)[1] have an altered carbohydrate composition when compared to those of age-, race-, and sex-matched controls (Scanlin & Glick, 1977). That is, the molar ratios of Fuc:NeuAc:Gal:Man were 1:3:4:2 and 1:4:7:5 in the membrane glycopeptides of CF and control fibroblasts, respectively. Since this group of glycopeptides is externally oriented toward the environment of the cells (Glick, 1979), it was of interest to isolate the glycoproteins from the growth medium and determine if an alteration exists also in these glycoproteins.

We report here methods for the isolation and a partial characterization of high molecular weight glycoproteins from the culture medium of human skin fibroblasts. A comparison of these glycoproteins from the medium after growth of CF and matched control fibroblasts is presented.

Materials and Methods

Materials. Sodium heparin was purchased from SchwarzMann; CNBr-activated Sepharose and heparin–Sepharose were from Pharmacia. L-[5,6-$^3H_2$]Fucose (60 Ci/mmol), L-[1,5,6-$^3H_3$]fucose (3 Ci/mmol), D-[6-^{3}H]glucosamine (19 Ci/mmol), L-[U-^{14}C]proline (294 mCi/mmol), and

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[1] Abbreviations: CF, cystic fibrosis; FCS, 10% fetal calf serum; NaDodSO$_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

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L-[U-^{14}C]lysine (300 mCi/mmol) were obtained from New England Nuclear Corp. Gelatin and fibronectin were obtained from Sigma, and Pronase was from Calbiochem; gas–liquid chromatography supplies were from Supelco. Tissue culture medium and FCS were from Flow Laboratories.

Cell Growth and Medium Preparation. Skin fibroblasts were obtained from CF patients and controls which were healthy age-, race-, and sex-matched individuals (Scanlin et al., 1977). All cultures used for these experiments were between passages 9 and 15. The matched cells were seeded at 1×10^6 cells and were grown at the same time and were of the same passage for the comparative experiments. The details of cell growth have been described (Scanlin et al., 1977; Scanlin & Glick, 1978). In all cases, the cultures were free of *Mycoplasma* (Levine & Becker, 1976). The radioactive monosaccharides L-[^{3}H]fucose or D-[^{3}H]glucosamine (5 μCi per 75-cm^2 flask) or L-[^{14}C]proline (5 μCi per 75-cm^2 flask) or L-[^{14}C]lysine (5 μCi per 75-cm^2 flask) were added to the medium 48 h before harvest unless otherwise stated. In some cases, the medium containing 10% FCS was removed and replaced with serum-free medium containing L-[^{3}H]fucose for 48 h before harvest. The medium was removed from the monolayer cultures, chilled, and centrifuged at 800*g* for 15 min, and the supernatant material was held at −30 °C until further processed.

When the growth experiments were terminated after removal of the medium, the cells were harvested as described (Scanlin & Glick, 1978), and aliquots were removed to determine the cell number, radioactivity, and protein content (Lowry et al., 1951). In all cases, the growth of the cells which were compared was within 10% of each other in cell number. The cells, after harvest, were 93–97% viable as measured by the exclusion of trypan blue. As reported previously (Scanlin & Glick, 1978), the growth rate and protein content per cell were similar for CF and control fibroblasts.

Preparation of Glycoproteins. All procedures were performed in the presence of 0.1% toluenesulfonyl fluoride dissolved in 2-propanol.

(*A*) *Precipitation with Heparin.* Medium containing serum (50 mL) or serum-free medium (100 mL) was dialyzed against water at 5 °C for 28 h with one change, lyophilized, and resuspended in 2 mL of sterile distilled water. A 4-mL sample of 0.1 M acetate buffer, pH 5.5, containing 6.0 mg of sodium heparin (154 units/mg) was added and held at 5 °C for 17 h. After centrifugation, 6500*g* for 20 min, the pellet was suspended in 0.5 mL of 0.1 M sodium phosphate buffer, pH 7.0, and held at 5 °C for 17 h. After recentrifugation, the pellet was resuspended in 0.5 mL of the phosphate buffer and held at 5 °C for 3 h. The pellet obtained after centrifugation at 6500*g* for 20 min was solubilized in 0.2 mL of a 0.05% solution of $NaDodSO_4$, and aliquots were removed for radioactive counting and protein determination (Lowry et al., 1951).

(*B*) *Cryoprecipitation.* Medium was treated exactly as described under Precipitation with Heparin and precipitated in the cold but without the addition of heparin.

(*C*) *Ammonium Sulfate Precipitation.* Medium (10 mL) was precipitated with 70% ammonium sulfate for 3 h at 5 °C. The pellet, separated by centrifugation (6500*g* for 20 min), was solubilized in 0.2 mL of a 0.05% solution of $NaDodSO_4$, and aliquots were removed for radioactive counting and protein determinations.

(*D*) *Heparin–Sepharose Column.* Medium (5 mL) was passed 5 times over a column (8 mm, disposable) containing 1 mL of heparin–Sepharose. The column was washed previously with 50 mL of 2 mM phosphate buffer, pH 7.4, in 0.05 M NaCl. After 1 h at room temperature, the column was washed with the buffer again and was eluted with 5 mL of 2 mM phosphate buffer in 1.0 M NaCl. Fractions of 1 mL were collected, and the radioactivity was determined by scintillation counting. Fractions were combined according to radioactivity, lyophilized, and suspended in a 0.05% solution of $NaDodSO_4$.

Gelatin–Sepharose Column. Gelatin was coupled to CNBr-activated Sepharose 4B (Iverius, 1971). After the mixture was washed in 2 mM Tris-HCl, pH 8.5, 9 mL of gelatin–Sepharose slurry was poured into a column (10 × 25 mm). Radioactive glycoproteins, precipitated from 50 mL of medium with heparin, were applied to the column in 9 mL of 2 mM Tris-HCl, pH 8.5, and after 17 h were eluted with 15 mL of 0.1 M NaCl, 1 M NaCl, and 8 M urea, respectively, in 2 mM Tris-HCl, pH 8.5. Fractions were combined according to radioactivity, dialyzed against water or 0.1 M acetic acid, and lyophilized.

Characterization of the Glycopeptides. The material precipitated with heparin (2500 cpm) was digested exhaustively with Pronase as described (Glick, 1979). The Pronase-digested material in a total volume of 0.6 mL was diluted with 0.2 mL of separation buffer (0.1 M Tris-HCl, pH 8.9, containing 0.1% $NaDodSO_4$, 0.01% EDTA, and 0.1% mercaptoethanol) and applied to a column (100 × 0.6 cm) of Sephadex G-50. Fractions (0.8 mL) which were eluted with separation buffer were collected and suspended in Triton-based scintillation fluid, and the radioactivity was counted in a scintillation counter. The data were calculated and graphed by computer (Glick, 1979).

Gel Electrophoresis. Polyacrylamide slab gels (7–14%) in 0.1% $NaDodSO_4$ under denaturing conditions were used as described (Laemmli, 1970). The gels were stained with Coomassie brilliant blue and either scanned in the Zeiss spectrophotometer using the gel scan attachment and automatic recorder or dried, photographed, and cut in 1.5-mm slices for determination of the radioactivity by scintillation counting (Flowers & Glick, 1980). Molecular weights were determined by comparison with fibronectin (M_r 220 000), rabbit skeletal muscle myosin (M_r 200 000), RNA polymerase (M_r 165 000 and 155 000), *E. coli* β-galactosidase (M_r 130 000), bovine serum albumin (M_r 68 000), ovalbumin (M_r 43 000), and chymotrypsinogen (M_r 23 000).

Carbohydrate Analysis. The material which was precipitated with heparin or which was eluted from the heparin–Sepharose column was dissolved in a 0.05% solution of $NaDodSO_4$ and, in the presence of the appropriate buffer, digested exhaustively with Pronase (Glick, 1979). The radioactive glycopeptides were separated by filtration over Sephadex G-25 in water and lyophilized. The glycopeptides were hydrolyzed in 0.02 N HCl over Dowex 50X2 H^+ (200–400 mesh) at 100 °C for 42 h and the alditol acetate derivatives prepared (Lehnhardt & Winzler, 1968). Quantitation of the monosaccharides was by gas–liquid chromatography with 2-deoxyglucose as the internal standard (Glick, 1974). Sialic acid was determined on the heparin-precipitated material after hydrolysis with 0.1 N H_2SO_4 for 1 h at 80 °C by a modification of the thiobarbituric acid assay (Glick, 1979).

Results

Glycoproteins and Proteins from the Medium. Glycoproteins were obtained by four different procedures from the medium of CF or control skin fibroblasts made radioactive with L-[^{3}H]fucose. The glycoproteins were subjected to polyacrylamide slab gel electrophoresis under denaturing con-

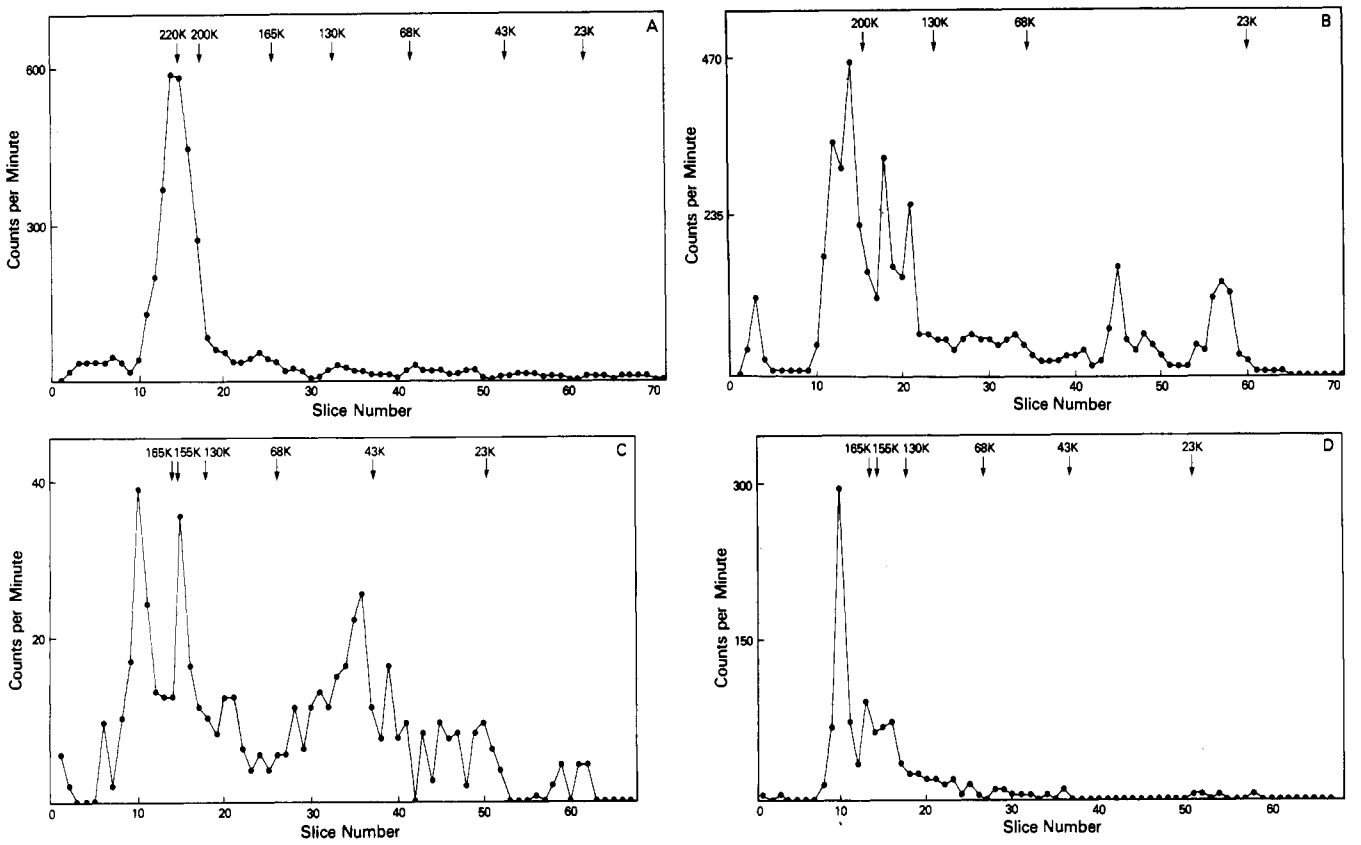

FIGURE 1: Profile of radioactive glycoproteins from human fibroblast culture medium separated by 7–14% gradient polyacrylamide gel electrophoresis under denaturing conditions. CF or control skin fibroblasts were grown for 5 days and labeled metabolically for 48 h with L-[^{3}H]fucose in medium containing FCS. The medium was removed and partially purified by (A) precipitation with heparin, (B) a heparin–Sepharose column, (C) precipitation with 70% ammonium sulfate, or (D) cryoprecipitation. All details are described under Materials and Methods.

ditions. Figure 1 shows that in all cases radioactive glycoproteins of M_r 220 000 or greater were obtained, but the degree of purity depended upon the procedure used. Precipitation with sodium heparin yielded the most pure preparation since only one major radioactive glycoprotein was obtained (Figure 1A). In contrast, the fractions obtained from the heparin–Sepharose column consistently showed additional radioactive glycoproteins (Figure 1B). Similarly, precipitation with ammonium sulfate provided a complex array of glycoproteins (Figure 1C). Cryoprecipitation (Figure 1D) yielded a major glycoprotein and additional minor radioactive glycoproteins of M_r 180 000–145 000. All of these molecular weights were approximate and were assigned with the use of skeletal muscle myosin, RNA polymerase, or in some cases fibronectin as markers.

The radioactive profile of the material precipitated with heparin (Figure 2A) or obtained from the heparin–Sepharose column (Figure 2B) when the fibroblasts were metabolically labeled with radioactive amino acid precursors showed that the major protein detected corresponded in size to that of the fucose-labeled material. The same profile was obtained with either [^{14}C]proline or [^{14}C]lysine as the amino acid precursor. Similar to the fucose-labeled glycoproteins, the amino acid labeled material from the heparin–Sepharose column was more heterogeneous than that of the heparin-precipitated material.

The yield of radioactive glycoproteins per milliliter of medium was 6 times less when the fucose-containing glycoproteins were precipitated by heparin than the yield obtained by ammonium sulfate precipitation and 10 times less than that obtained from the heparin affinity column. However, the radioactivity was greater than 90% at the approximate molecular weight, 220 000, so the heparin precipitation procedure was utilized to obtain material for further characterization. The yield of radioactivity from cryoprecipitation was 10 times less than that from heparin precipitation.

Table I: Carbohydrate and Protein Content of the Heparin Precipitate from Fibroblast Medium with and without FCS[a]

composition	no FCS	10% FCS	10% FCS blank
Fuc (pmol/mL of medium)	53	33	0
Man (pmol/mL of medium)	267	351	70
Gal (pmol/mL of medium)	240	335	40
Glc (pmol/mL of medium)	324	271	400
GlcN (pmol/mL of medium)	164	327	120
protein (μg/mL of medium)	6	36	21
radioactivity (cpm/mL of medium)	63	200	0

[a] Skin fibroblasts were grown for 5 days in medium containing FCS and for an additional 48 h in medium of the same ingredients (10% FCS) or with no FCS. A blank of medium containing FCS was incubated for 48 h. All three contained L-[^{3}H]fucose (5 μCi per flask). The media were precipitated with heparin. See Materials and Methods for all details.

Heparin-Precipitated Material from Medium with and without FCS. The radioactive glycoproteins prepared by heparin precipitation were of similar molecular weight whether or not the medium in which the fibroblasts were grown contained FCS for 48 h prior to harvest (Figure 1A). The heparin-precipitated material contained 3–8 times less radioactivity when the cells were cultured on serum-free medium rather than on serum-containing medium for 48 h prior to harvest. In addition, the amount of protein precipitated was 5 times less than that precipitated from medium with FCS (Table I).

When the heparin-precipitated material was hydrolyzed and the monosaccharide content was determined, the difference

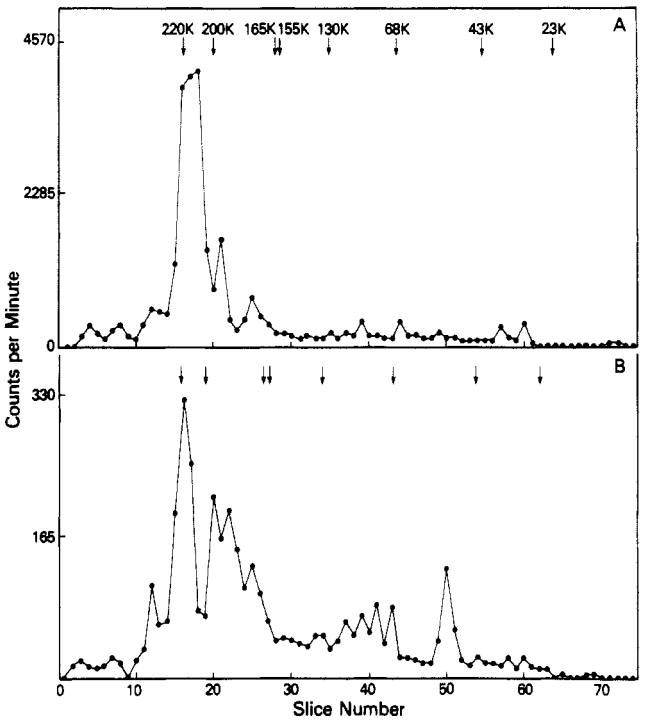

FIGURE 2: Profile of radioactive proteins from human fibroblast culture medium separated by 7–14% gradient polyacrylamide gel electrophoresis under denaturing conditions. CF or control skin fibroblasts were grown for 5 days and labeled metabolically for 48 h with radioactive amino acids. The medium was (A) labeled with L-[^{14}C]lysine and precipitated with heparin or (B) labeled with L-[^{14}C]proline and purified on a heparin–Sepharose column. All details are described under Materials and Methods.

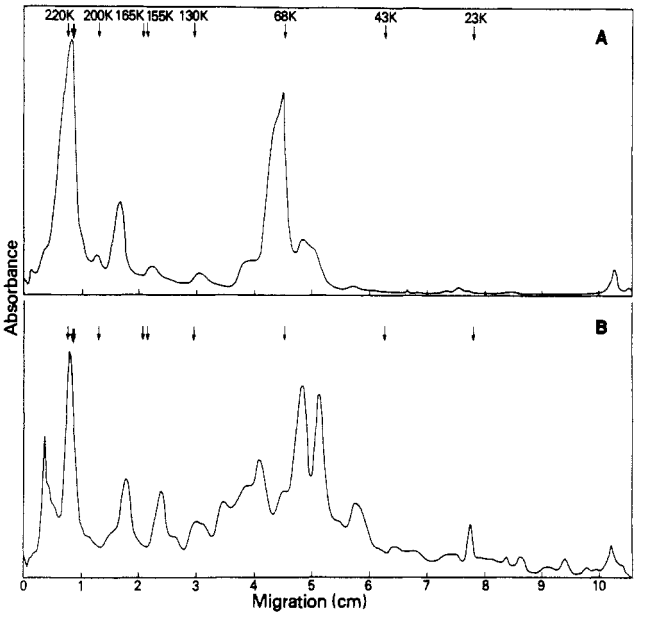

FIGURE 3: Profile of the proteins from fibroblast culture medium separated by polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue. Medium was (A) precipitated with heparin or (B) purified on a heparin–Sepharose column. See legend to Figure 2.

with or without FCS was not striking with the exception of the fucose content (Table I). An increase in fucose content in the precipitated medium without FCS was observed. When FCS-containing medium in which fibroblasts were not grown was precipitated with heparin as a blank, some monosaccharides from the serum glycoproteins were recovered when analyzed by gas–liquid chromatography. When this monosaccharide content was subtracted from the FCS medium after fibroblast growth, even less difference was discernible in the carbohydrate content of the precipitated medium with or without FCS with the exception of fucose (Table I). No fucose was detected in the blank.

The fact that some of the proteins which were precipitated with heparin were from FCS of the serum-containing medium is shown further by comparing Figures 2A and 3A. Figure 3 shows the profile of proteins stained with Coomassie brilliant blue. Most of the proteins were exogenous since no radioactive label was incorporated when L-[^{14}C]lysine or -proline was used as a radioactive precursor (Figure 2). The blank of medium with 10% FCS incubated in the presence of L-[^{3}H]fucose for 48 h and precipitated with heparin contained only nonradioactive proteins (Table I) which were detected by Coomassie blue staining after polyacrylamide gel electrophoresis.

The profile of the proteins stained with Coomassie brilliant blue obtained by heparin precipitation of medium containing no FCS showed in addition to the major band, M_r 220 000, two other major bands, M_r 175 000 and 68 000.

Proteins Obtained by the Other Procedures. The heparin affinity column yielded additional fucose-containing glycoproteins (Figure 1B) and radioactive proteins (Figure 2B) but was similar to the heparin-precipitated material in that some of the proteins were not radioactive when obtained from medium containing FCS (Figure 3B). Particularly prominent was a protein greater than M_r 220 000 (Figure 3B). As with the fucose-containing glycoproteins, precipitation with heparin had fewer proteins than the heparin affinity column.

In addition to the radioactive glycoproteins in the cryoprecipitate (Figure 1D) and the ammonium sulfate precipitate (Figure 1C), other proteins were present also in these fractions. The major protein representing 50% or more of the material which stained with Coomassie brilliant blue was M_r 68 000. Fifteen or more other protein bands were also present. Similar to the radioactive profile (Figure 1C), the ammonium sulfate precipitated material was most heterogeneous.

Further Characterization of the Heparin Precipitate. The radioactive glycoprotein-containing fraction which was precipitated with heparin from medium containing FCS was further purified on a gelatin–Sepharose column. The column was first washed with 0.1 M NaCl, and 40% of the radioactivity was eluted. Additional radioactivity (20%) was eluted with 1 M NaCl. The column then was eluted with 8 M urea, and 40% of the total radioactivity was recovered. The urea and 0.1 M NaCl fractions were dialyzed against water, lyophilized, and analyzed by polyacrylamide gel electrophoresis under denaturing conditions. The fraction which was eluted with 8 M urea separated into a doublet of M_r 210 000 and 220 000 using rat skeletal muscle myosin as a reference; the fraction which was eluted with 0.1 M NaCl contained a radioactive glycoprotein, M_r 187 000. Minor amounts of several other nonradioactive proteins were still present in both of these fractions as detected by staining with Coomassie brilliant blue. Using the Ouchterlony technique of immunodiffusion with undiluted antiserum to cold insoluble globulin, the 8 M urea fraction was nonreactive after concentration and dialysis in an Amicon A25. At the same time, the heparin-precipitated material showed a precipitin band.

In contrast to the medium components precipitated with heparin, when the starting material was from the ammonium sulfate precipitation of the medium only 14% of the radioactivity was eluted with 8 M urea from the gelatin–Sepharose column. Most of the radioactivity was in the fall-through of the affinity column. When this radioactive material was reapplied to a fresh column, it again ran through, showing that the column was not overloaded and that the ammonium sulfate precipitable material was heterogeneous.

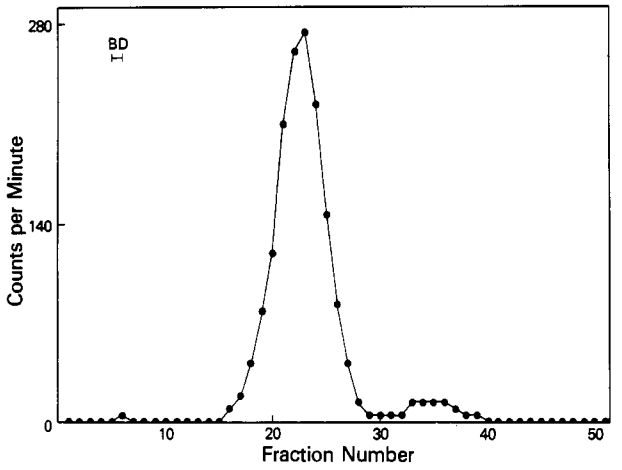

FIGURE 4: Chromatography on Sephadex G-50 of the Pronase-digested glycoproteins from the fibroblast culture medium. The glycoproteins were made radioactive by growth of the CF or control skin fibroblasts in medium containing L-[^{3}H]fucose. The medium was precipitated with heparin and subsequently digested with Pronase. All details are described under Materials and Methods.

Table II: Fucose Incorporation into Heparin Precipitates[a]

days in culture	fibroblast	radioactivity: cpm/mg of protein[b]	radioactivity: % of control
7	CF	7640	79
	C	9670	100
9	CF	9220	65
	C	14150	100

[a] CF and control (C) fibroblasts were grown for 72 h in medium containing 10% FCS and for an additional 48 h (day 7) or 96 h (day 9) under the same conditions in the presence of L-[^{3}H]fucose (5 μCi per flask). Prior to harvest, the radioactive medium was removed and incubation continued for 48 h in medium without 10% FCS, making a total of 7 or 9 days in culture. This medium was precipitated with heparin as described under Materials and Methods. [b] Average of two experiments on two separate matched cell lines. All determinations were done in duplicate.

The material labeled with L-[^{3}H]fucose and precipitated by heparin was digested exhaustively with Pronase and examined by gel permeation chromatography as described previously (Glick, 1979). Only one size class of glycopeptides was obtained (Figure 4). This group of glycopeptides was similar by gel permeation chromatography to the peripheral glycopeptides isolated previously from the surface of the human fibroblasts by a mild and controlled trypsinization procedure (Scanlin & Glick, 1978).

Radioactive D-[^{3}H]glucosamine was also used as the carbohydrate precursor. The radioactive profile obtained after polyacrylamide gel electrophoresis of the heparin-precipitated material was similar to that shown in Figure 1A for the fucose-containing material.

Comparisons of CF with Control Medium Glycoproteins. A total of eight matched sets were examined in the experiments thus far described. In all cases, similar results were obtained by polyacrylamide gel electrophoresis and gelatin–Sepharose chromatography with medium from CF or control fibroblasts. Several differences were noted, however, and these included the following.

(*1*) L-[^{3}*H*]*Fucose Incorporated.* The heparin-precipitable material from the medium after growth of CF fibroblasts contained less radioactivity per milligram of precipitated protein than that of the control when L-[^{3}H]fucose was used as the glycoprotein precursor (Table II). Fibroblasts were grown on medium containing FCS for 3 days and then made radioactive by growth in the presence of L-[^{3}H]fucose (5 μCi per 75-cm^2 flask) for 48 h and divided into two groups. For the first group, the radioactive medium was removed. The monolayer was washed, and growth was continued for 48 h in medium without FCS. The medium was removed and precipitated with heparin (Table II, day 7). Growth in the radioactive medium was continued for 48 h for the second group. The medium was removed, the monolayer was washed, and growth was continued for 48 h in medium without FCS. This medium was precipitated with heparin (Table II, day 9). In both cases, when the medium contained no FCS (day 7 and day 9 media) less radioactivity per milligram of protein (79% and 65%, respectively) was found in the heparin-precipitated CF medium than in that of the control.

Table III: Characterization of the Heparin Precipitate from Media of Confluent CF and Control Fibroblasts

composition	days in culture[a]: 7 CF	7 C	9 CF	9 C
Fuc (nmol/mg of protein)	7	2	4	8
Man (nmol/mg of protein)	43	21	43	45
Gal (nmol/mg of protein)	48	20	43	38
Glc (nmol/mg of protein)	29	16	69	51
GlcN (nmol/mg of protein)	37	19	40	26
sp act. (cpm/nmol of Fuc)	4460	5700	2285	1935
radioactivity (cpm/10^6 cells)	3180	1340	300	750

[a] CF and control (C) fibroblasts were grown to day 5, and medium containing FCS and L-[^{3}H]fucose was added for 48 h. The medium was removed and precipitated with heparin (day 7), and incubation of the cells was continued for 48 h (day 9) in medium with no FCS or radioactivity. All details are described under Materials and Methods.

(*2*) *Protein Content.* When the CF and control fibroblasts were grown on medium with or without FCS, the amount of protein precipitated with heparin was less from the medium of the CF cells than from that of the control cells in six out of eight matched sets.

(*3*) *Carbohydrate Content.* In order to determine if the secreted components had an altered ratio of fucose to the other monosaccharides as seen in the glycopeptides obtained from the fibroblast membranes by mild trypsin treatment (Scanlin & Glick, 1978), the monosaccharide composition of the material precipitated by heparin from medium of cells grown for 7 days was examined from several matched sets of fibroblasts. The material precipitated from the CF medium containing FCS had a higher ratio of fucose to galactose, i.e., 1:6 as compared to 1:10 of the control medium (Table III). Therefore, the material secreted by these CF fibroblasts reacted differently with the components of FCS or else secreted glycoproteins of different composition in the presence of FCS when compared to control fibroblasts. The fact that the variation was not due to serum glycoproteins per se is shown in Table I where only minor quantities of the monosaccharides were precipitated from medium incubated as a blank. No fucosyl residues were recovered from the blank medium containing FCS, so a high fucose content in the precipitated glycoproteins may be a characteristic of the cell-related material.

This difference in fucose content was not apparent in the medium components which were eluted with 2 mM phosphate buffer in 1.0 M NaCl from the heparin–Sepharose column. Although less fucose per milligram of protein was obtained, there was 3–6 times more mannose and galactose. The monosaccharide composition in the order Fuc, Man, Gal, and Glc was 4, 138, 179, and 63 and 5, 125, 154, and 32 nmol/mg of protein from the media of CF and control fibroblasts, respectively. Thus, no difference between the CF and control

medium was apparent in this material with the exception that the glucose content of the CF material was approximately 2 times higher than that of the control when FCS was present. The presence of a high glucose content in the 10% FCS blank (Table I) makes this difference difficult to interpret.

(*4*) *Sialic Acid Content.* A comparison of the sialic acid content from the heparin-precipitated medium revealed that the CF material contained more sialic acid than that of the control, 53 and 28 nmol/mg of protein, respectively. For this experiment, a matched set of fibroblasts was grown to confluency (day 10) and then held 2 days in medium with no FCS. This medium was precipitated with heparin.

As with other monosaccharides, the sialic acid content of the heparin precipitate of the medium without 10% FCS increased in amount with confluency. The sialic acid content of the heparin-precipitated medium of CF cells grown for 12 days was increased 5-fold when compared to the medium of day 4 cells, 47 pmol/10^4 cells compared to 9 pmol/10^4 cells, respectively. The confluent cells were grown for 2 days prior to harvest without FCS whereas the rapidly growing cells were in the presence of FCS. Even with this difference, the amount of sialic acid was markedly increased.

(*5*) *Turnover of Heparin-Precipitated Material.* Medium was precipitated by heparin after growth of the CF and control fibroblasts to day 7 in the presence of FCS. The cells were allowed to incorporate radioactive L-[^{3}H]fucose from day 5 to day 7. After the radioactive medium was removed for precipitation with heparin (Table III, day 7), the fibroblasts were washed and incubated for an additional 2 days in medium with no FCS and no L-[^{3}H]fucose (Table III, day 9).

Table III shows the drop of radioactivity per nanomole of fucose from day 7 to day 9 of the heparin-precipitated medium. The CF material, although not strikingly different from the control material, had a decrease in specific activity at day 9 which was 50% of that at day 7, whereas the control material decreased from 5700 to 1940 cpm/nmol of fucose.

These results were complicated by the fact that there was a dramatic decrease in radioactivity precipitated with heparin from the CF medium from day 7 to day 9 when expressed per 10^6 cells (3180 to 300 cpm/10^6 cells). On the other hand, the control material was decreased by less than 50% (1340 to 750 cpm/10^6 cells). In both cases, CF and control, the amount of protein which was precipitated was also decreased. This was expected because of the difference in protein content of medium with and without FCS (Table I).

Discussion

Four methods were presented to obtain glycoproteins from the culture medium of human fibroblasts. One of these methods, precipitation by heparin, was utilized to obtain a high molecular weight glycoprotein which under reducing conditions had an apparent molecular weight of greater than 200 000. Whether or not this glycoprotein was secreted by the cells or resulted from the shedding of membrane glycoproteins has yet to be determined. This glycoprotein has some similarity to fibronectin (Vaheri & Mosher, 1978).

Among the similarities to fibronectin in addition to the reaction with heparin and precipitation in the cold was the binding to a gelatin–Sepharose column (Ruoslahti & Vaheri, 1975). With use of the heparin-precipitated material from the medium which was already purified greater than 1000-fold, further fractionation of the radioactive glycoproteins was obtained by sequential elution with NaCl and urea from a gelatin–Sepharose column. Fibronectin was eluted with urea (Ruoslahti & Vaheri, 1975), and in the studies reported here, fucose-containing glycoproteins, M_r 220 000 and 210 000, were eluted with urea. Other glycoproteins which migrated on polyacrylamide gels, M_r 187 000, under reducing conditions were eluted from the column of gelatin–Sepharose with 0.1 M NaCl. The latter glycoprotein could be a degradation product since it was 40% of the total radioactivity and was not observed in the heparin precipitate which was the starting material for this column (Figure 1A). These apparent changes in the electrophoretic mobility on the polyacrylamide gels were reminiscent of the protease (Gold et al., 1979) or the sulfhydryl cleavage (Wagner & Hynes, 1979) products of fibronectin. It is not clear at this time whether or not the radioactive glycoprotein(s) is (are) fibronectin. The lack of binding of the material purified by the gelatin–Sepharose column with antiserum to cold-insoluble globulin and the presence of a high ratio of fucose to other monosaccharides (Table III) are not characteristic of fibronectin. However, there appears to be a family of fibronectins with species (Kobata, 1979) as well as cellular and plasma (Rouslahti & Vaheri, 1975) differences. The structure of the carbohydrate portion of fibronectin, as reported by Takasaki et al (1979), differed from that reported previously by Carter & Hakomori (1979). From the results of our studies which select high molecular weight fucose-containing glycoproteins from the medium, we are led to conclude that the family of high molecular weight glycoproteins may be even more heterogeneous.

The differences in amounts and kinds of glycoproteins purified by precipitation with heparin or the heparin affinity column were remarkable. The method of precipitation yielded a purer fraction, although the heparin column gave a more representative spectrum of glycoproteins (compare panels A and B of Figure 1). The latter method would be of use as a first step to isolate medium components whereas the former method increased the yield of a more purified product.

This study was originated to look for differences in the fucose-containing glycoproteins of the medium of CF fibroblasts when compared to the controls as part of the study examining abnormal fucose metabolism in CF (Scanlin & Glick, 1978, 1980). The fact that medium from CF fibroblasts has activity in assays defining the CF factor (Bowman et al., 1973) also makes a study of the fibroblast medium worthwhile. Related facts are that heparin reversed the ciliostatic ability of CF serum and parotid secretions (Dogget & Harrison, 1973), and glycoproteins precipitated by heparin from CF serum had a different carbohydrate composition than those of serum from control individuals (Pearson & Lubin, 1979; T. F. Scanlin and M. C. Glick, unpublished results).

A number of differences were noted when the heparin-precipitated material from the CF medium without fetal calf serum was analyzed and compared to that of the matched controls. These differences showed that the CF material contained (1) less protein and carbohydrate, (2) 30–35% less radioactivity per milligram of protein, (3) lowered fucose content, (4) higher sialic acid content in confluency, and (5) lower turnover when expressed as counts per minute per nanomole of fucose.

In contrast to the low fucose content of the heparin-precipitated material obtained from CF medium without FCS, when FCS was present, a higher fucose content was observed in the CF material than that of the control. In addition, when the medium from the CF fibroblasts contained fetal calf serum, the heparin precipitate had more radioactivity. These compositional differences could reflect a differential response of the CF and control fibroblasts to growth in medium with or without FCS. Alternately, these differences could represent a differential recruitment of the other glycoproteins during

the precipitation of serum-containing medium.

It is of interest that there was an increased amount of sialic acid per milligram of protein in the heparin precipitate from medium without FCS of highly confluent CF fibroblasts. Similar material from confluent cells (Table III) showed a decreased fucose content. Previous results showed that the membrane glycoproteins of CF fibroblasts had an increased ratio of fucose to sialic acid when compared to the controls (Scanlin & Glick, 1977). The reverse effect, lowered fucose content and higher sialic acid content in the media, as reported here could relate to the process of secretion. Although additional comparisons are necessary before these fucose to sialic acid ratios can be generalized, it supports the work of Dische et al. (1959) that CF secretions had an altered fucose to sialic acid ratio. Further support for differences in fucosylation was the increase in fucose of the heparin precipitate of day 7 medium of the CF fibroblasts and the lowered turnover of radioactive fucose (Table III).

An altered distribution of α-L-fucosidase in CF has been suggested from other studies (Scanlin et al., 1977, 1979). This concept has been extended to another cell type by Maler et al. (1981), who demonstrated a decreased activity of α-L-fucosidase in transformed CF lymphocytes. In addition, α-L-fucosidase purified from CF liver has an altered carbohydrate composition (Alhadeff et al., 1978) and decreased affinity to concanavalin A (Alhadeff & Watkins, 1979). An altered fucose content of CF meconium glycoproteins (Clamp & Gough, 1979), secreted intestinal mucins (Wesley et al., 1981), α_2-macroglobulin (Ben-Yosef et al., 1980), and other serum glycoproteins (Pearson & Lubin, 1979) has also been reported. All of these findings combined with the original observation of Dische et al (1959) suggest a relationship of fucosyl residues to the genetic defect. Further support for this concept comes from the fact that several of these differences have been described in cultured cells, which are removed from the secondary effects of the disease.

The specificities inherent in glycosylation mechanisms and the biological phenomena which are determined by carbohydrate residues are currently among the foremost problems in biology (Neufeld & Ashwell, 1980). Further studies of the subtle differences reported here in the glycoproteins of CF fibroblast medium when compared to those of the matched control medium may help not only to elucidate the basis of the defect in CF but also to contribute to understanding the role of glycosylation.

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