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Comparison of the Structures of Human Fibronectin and Plasma Cold-Insoluble Globulin

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Human amniotic fluid fibronectin and plasma fibronectin (cold-insoluble globulin) are indistinguishable both immunologically and by amino acid composition. Cyanogen bromide and tryptic peptides also suggest substantial structural homology. However, carbohydrate analysis has demonstrated additional saccharides in fibronectin and an overall increase in carbohydrate content relative to cold-insoluble globulin. Furthermore, limited proteolytic cleavage of the two proteins indicates differences in primary structure or in conformation. Using affinity-purified antibodies to cold-insoluble globulin, a glucosamine-labeled pronase-resistant component, probably proteoglycan, was found to coprecipitate with fibronectin, suggesting an association between these two macromolecules in the connective tissue matrix.

Key words: fibronectin, cold-insoluble globulin, carbohydrate content, proteoglycan, proteolytic cleavage

Fibronectin is a high-molecular-weight glycoprotein that is present in a variety of connective tissues including basement membranes [1]. Fibronectin is also synthesized and secreted by cells in culture and is frequently deposited on the cell surface and in the extracellular matrix produced by these cells [2–6]. A circulating glycoprotein, cold-insoluble globulin (CIG), bears extensive structural and immunologic similarities to fibronectin synthesized by cells and is sometimes referred to as plasma fibronectin [7–10]. Other body fluids, such as amniotic fluid, also contain fibronectin [4, 11].

Fibronectin has been shown to function in cell adhesion [8, 12], in maintenance of cell shape [13, 14] and in cellular movement [15]. The protein interacts with a number of components such as collagen [16–18] and fibrin [19] and can become crosslinked to fibrinogen through the action of plasma transglutaminase [20]. The association of fibronectin with certain cell-surface macromolecules may be necessary for the cell surface functions mediated by fibronectin.

Our objective was to study the similarities and possible differences between CIG and cellular and amniotic fluid fibronectins. Despite the very extensive structural and functional similarities between fibronectin and CIG, a number of findings suggest that the proteins are not identical. Reproducible differences in migration on sodium dodecyl

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sulfate (SDS) gels have been reported; CIG can be resolved as a closely spaced doublet, whereas fibronectin isolated from amniotic fluid, cell culture medium, and cell layer migrates more slowly as a broad band [4, 21–23]. CIG is somewhat less effective in promoting the attachment of virally transformed hamster fibroblasts to a substratum [21], and is considerably less effective in hemagglutination of fixed erythrocytes [24] and in restoring a more normal morphology to transformed cells [24]. In this report we present evidence that fibronectin isolated from human amniotic fluid [4] differs from CIG in its carbohydrate content and in the peptide pattern produced by limited cleavage with two proteases. Preliminary evidence for the interaction of cellular fibronectin with proteoglycans is also provided.

METHODS

Preparation of Cold-Insoluble Globulin

Cold-insoluble globulin was prepared from human plasma cryoprecipitates by a modification of the method described by Mosesson and Umfleet [25]. Frozen plasma was thawed in an ice bath at 0°C; the residue (cryoprecipitate) was centrifuged and dissolved in 0.2 M NaCl, 0.02 M imidazole buffer (pH 6.8) at room temperature. Glycine was added to a final concentration of 2.1 M, during which the temperature of the mixture dropped to 15°C. After the precipitate was removed by centrifugation at 15°C, the supernatant was diluted with an equal volume of cold distilled water and the proteins were precipitated by the addition of 20% v/v ethanol at -4° C. The mixture was maintained at -4°C for 30–60 min and centrifuged at 6,000g for 30 min. The pellet was dissolved in 0.04 M succinate-Tris buffer (pH 7.0) and the CIG was purified by chromatography on DEAE-cellulose using a linear gradient from 0.04–0.1 M succinate-Tris. When necessary, CIG prepared by DEAE-cellulose chromatography was purified further on Biorex-70 (BioRad), using a linear gradient of 0.1–0.3 M succinate-Tris (pH 6.8). CIG preparations produced a characteristic doublet on SDS-polyacrylamide gel electrophoresis when disulfide bonds were reduced. CIG was also prepared from plasma by affinity chromatography on gelatin-Sepharose [16]. A 0.5-mg portion of CIG was reduced and alkylated essentially as described by Crestfield et al [26] using 0.2 mCi [^{3}H] iodoacetic acid.

Preparation of Fibronectin

Second-trimester human amniotic fluid was centrifuged to remove debris, and EDTA (25 mM), phenylmethanesulfonyl fluoride (PMSF, 1 mM), and N-ethylmaleimide (10 mM) were added to minimize proteolysis. Solid ammonium sulfate was added to a final concentration of 20% (w/v) and the precipitate produced was removed by centrifugation and redissolved in 0.05 M Tris-HCl, 0.15 M NaCl (pH 7.5) containing 1 mM PMSF. Further purification was achieved by affinity chromatography on denatured collagen (gelatin)-Sepharose.

Preparation of Antibodies

Fibronectin or CIG (100–200 μg), dissolved in 1 ml phosphate-buffered saline and mixed with an equal volume of Freund's complete adjuvant, was used to immunize rabbits by subcutaneous injection at weekly intervals. Antisera were obtained 4 weeks after the start of the immunization program and after periodic booster injections.

Radioimmunoassays

Inhibition assays were performed as described previously [4] using ^{125}I-CIG or ^{125}I-fibronectin and rabbit antisera to these proteins. The concentration of inhibitor was determined using an absorption coefficient ($E^{1\%}_{1\ cm}$) at 280 nm of 13.

Immune Precipitation of Radiolableled Fibronectin

Rabbit anti-human CIG antibodies were purified by affinity chromatography on CIG linked to Sepharose. Bound IgG was eluted with 0.2 M glycine adjusted to pH 2.8 with HCl and dialyzed against phosphate-buffered saline. Affinity-purified antibody (0.6 mg/ml) was added to radiolabeled culture medium or to cell layer extracts and incubated for 1 h at room temperature. Sheep anti-rabbit gamma globulin antiserum was added and the mixture incubated at 4°C for 16 h. The precipitate was centrifuged and washed with 0.05 M Tris-HCl (pH 7.5). Reduction and alkylation of the immune precipitates was performed essentially as described previously [26].

Metabolic Labeling of Cells in Culture

Amniotic fluid-derived (AF) cells were prepared and maintained as previously described [4]. Cells were labeled with one of the following: 125 μCi/ml [^{35}S] cysteine-HCl, 50 μCi/ml ^{3}H-amino acid mixture, 50 μCi/ml [2 - ^{3}H] mannose, or 50 μCi/ml [1 - ^{14}C] glucosamine. Incubations were performed for 24 h in serum-free medium deficient in the corresponding sugar or amino acid. Culture medium was harvested into protease inhibitors [4]. The cells were first extracted with Hanks's balanced salt solution containing 1 M urea and 0.5 mM PMSF for 15 min at room temperature; cell layers were then suspended in 0.05 M sodium phosphate (pH 11) containing 1% Triton X-100, and were sonicated and centrifuged to remove cell debris. Culture medium, urea extracts, and cell layers were dialyzed into 0.05 M Tris-HCl, 0.15 M NaCl (pH 7.5).

Enzymatic Cleavage

Digestion of a mixture of [^{35}S] cysteine-labeled fibronectin and ^{3}H-CIG with trypsin (Worthington, TPCK) was performed in 0.1 M NH_4HCO_3 containing 1 mM $CaCl_2$ at 37° for 24 h using an enzyme-to-substrate ratio of 1 to 10 by weight, and the sample was lyophilized. Cation exchange chromatography of tryptic peptides of radiolabeled fibronectin and CIG was performed on a 0.9 - x 50 -cm column of sulphonated polystyrene (UR-30, Beckman) at 55° C using pyridine acetate buffers as previously described [27].

Digestion with cathepsin D (Sigma Chemical Co.) was performed at pH 3.5 and 30°C for 12 h in the presence of 0.2 mM PMSF, using an enzyme-to-substrate weight ratio of 1:250. Reactions were stopped by the addition of a 10-fold molar excess of pepstatin (Peptide Research Institute, Osaka, Japan). Digestion with mast cell protease, an enzyme with chymotrypsin-like specificity purified from rat peritoneal cavity (M. Everett and H. Neurath, in preparation), was performed at pH 7.5 and 30°C for 4 h using an enzyme-to-substrate weight ratio of 1:800. Enzymatic reactions were stopped and protein was precipitated by the addition of trichloroacetic acid (TCA) at 0° C to a final concentration of 10%. Precipitated proteins were prepared for SDS polyacrylamide slab gel electrophoresis by the addition of 0.2% SDS in 0.1 M Tris-HCl buffer (pH 6.8) with or without 50 mM dithiothreitol (DTT). Protein components in gels were stained with a 0.25% solution of Coomassie Brilliant Blue R and radioactive components were visualized by autoradiography [4].

Digestion of immune precipitated fibronectin with pronase (Calbiochem, grade B) was performed in 0.05 M sodium phosphate buffer (pH 7.5) at 37°C with an enzyme-to-substrate weight ratio of 1:30 for 60 h; at this point a second aliquot of enzyme was added and the digestion continued for 24 h. The samples were dialyzed and lyophilized. Chromatography on Sephadex G-50 superfine (Pharmacia) was performed using a 1.2 × 135 cm column eluted with 0.1 M ammonium bicarbonate. Cleavage with cyanogen bromide was performed in 70% formic acid at 30°C for 4 h using an equal ratio, by weight, of cyanogen bromide to protein.

Affinity Chromatography

Salt-soluble collagen, purified from lathyritic rat skin, was denatured by heating at 45°C for 30 min, and coupled to Sepharose CL4B (Pharmacia) using CNBr. Samples were applied to the column in the presence of protease inhibitors in 0.05 M Tris-HCl, 0.15 MNaCl, pH 7.5 at 4° C. Bound fractions were eluted with the above buffer containing 6 M urea.

Amino Acid Analysis

Lyophilized samples were hydrolyzed in vacuo with twice distilled hydrochloric acid at 108°C for 24 h and amino acid compositions were determined on a Durrum D500 analyzer. No corrections were made for hydrolytic losses of individual amino acids. Tryptophan was determined as described by Hugli and Moore [28].

Carbohydrate Analysis

Both fibronectin and CIG were further purified from possible carbohydrate-containing contaminants by molecular sieve chromatography on a column of controlled pore glass (700 Å, Electro Nucleonics Inc.) equilibrated in 0.1% SDS, 1 M urea, 0.025 M Tris-HCl, 0.2 M glycine buffer (pH 8.8). SDS bound to protein was removed by sequential dialysis against 80% acetone and water. Samples were dried to a constant weight and hydrolyzed in 80% acetic acid, 0.5 M H_2SO_4, for 8 h at 80°C and carbohydrate composition was determined by gas-liquid chromatography of the alditol acetate derivatives. Sugars were identified by their positions of elution using an internal standard of inositol; their identification was confirmed by mass spectrometry. Sialic acid was determined by the thiobarbituric acid method using a spectrofluorometric assay [29].

RESULTS

The immunologic cross-reaction of fibronectin and CIG was studied by radioimmunoassay. Purified amniotic fluid fibronectin and CIG inhibited the precipitation of iodinated CIG by anti-CIG antibodies in an identical fashion (Fig. 1). Comparable curves were obtained using culture medium from human amniotic fluid cells or skin fibroblasts as inhibitors of anti-CIG antibodies. Antiserum to amniotic fluid fibronectin was also completely inhibited by both fibronectin and CIG. These experiments indicate complete immunologic cross-reactivity of fibronectin, derived from amniotic fluid or from cells in culture, with plasma CIG.

The amino acid compositions of amniotic fluid fibronectin, culture medium fibronectin, and plasma CIG are shown in Table I. The high degree of similarity among these analyses suggests extensive structural homology between the proteins. Of note is the unusually high amount of threonine present in both proteins. A survey of the level of this amino acid

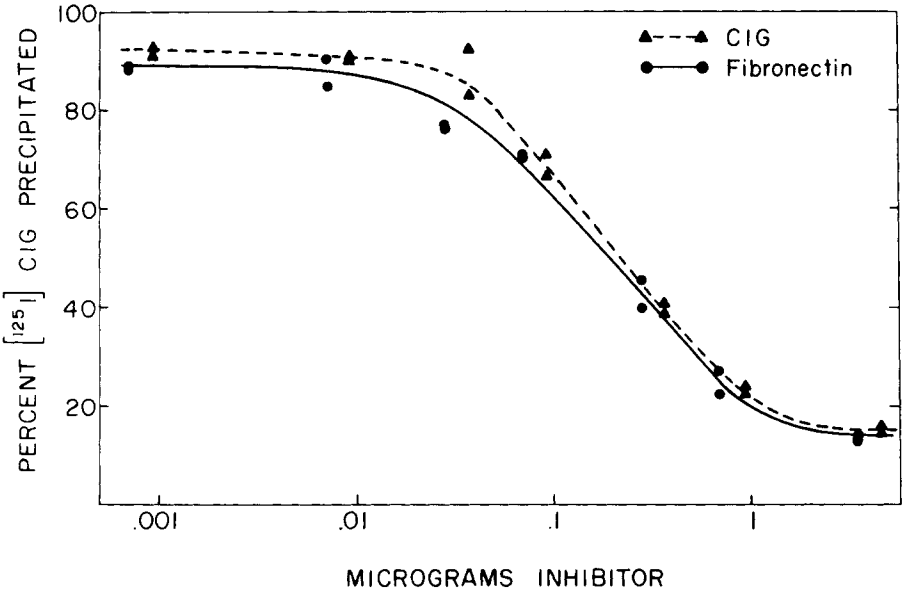

Fig. 1. Radioimmune inhibition curve. Increasing quantities of unlabeled CIG or fibronectin were used to inhibit the precipitation of [^{125}I]CIG by anti-CIG IgG in a double-antibody assay.

TABLE I. Amino Acid Compositions of Human CIG and Amniotic Fluid and Cellular Fibronectins

	Residues/1,000 residues		
	Plasma CIG	Amniotic fluid fibronectin	Cell culture medium fibronectin[a]
Aspartic acid	94	95	92
Threonine	106	101	106
Serine	74	78	78
Glutamic acid	120	122	118
Proline	93	92	71
Glycine	87	88	90
Alanine	42	43	47
Half-cystine[b]	24	25	23
Valine	76	73	77
Methionine	10	11	12
Isoleucine	42	42	45
Leucine	51	56	61
Tyrosine	39	36	40
Phenylalanine	23	25	27
Histidine	21	20	23
Lysine	35	32	37
Arginine	51	50	52
Tryptophan	15	11	ND

[a]From amniotic fluid-derived AF cells.
[b]Determined by performic acid oxidation.
ND: Not determined.

in other proteins shows that a value of about 10% of the total residues is exceeded only by silk fibroin and a few other glycoproteins.

We have previously shown that a cysteine-rich collagen-binding region from fibronectin and CIG migrates differently on SDS-PAGE [18]. Cation exchange chromatography of the tryptic peptides obtained from fibronectin and CIG, radiolabeled at cysteine residues, is shown in Figure 2. There is extensive correspondence and there are no major qualitative differences between the peptides derived from the two proteins, indicating that the cysteine-containing regions are structurally very similar in CIG and fibronectin. The fractions that were not retained by cation exchange chromatography (Fig. 2) were further analyzed by anion exchange chromatography on Dowex-1. The more negatively charged peptides from fibronectin and CIG were also observed to coelute (data not shown).

CIG and fibronectin were separately cleaved with cyanogen bromide and the resulting patterns were analyzed on 15% SDS-acrylamide gels and visualized by staining (data not shown). The pattern of peptides confirmed that extensive similarities exist between the two proteins, as indicated previously using digestion with trypsin. Only minor differences in the pattern of fibronectin- and CIG-derived peptides were apparent. Two proteases were also used to compare the products of partial proteolysis of fibronectin and CIG. The difference in electrophoretic migration between CIG and fibronectin is illustrated in Figure 3 (lanes

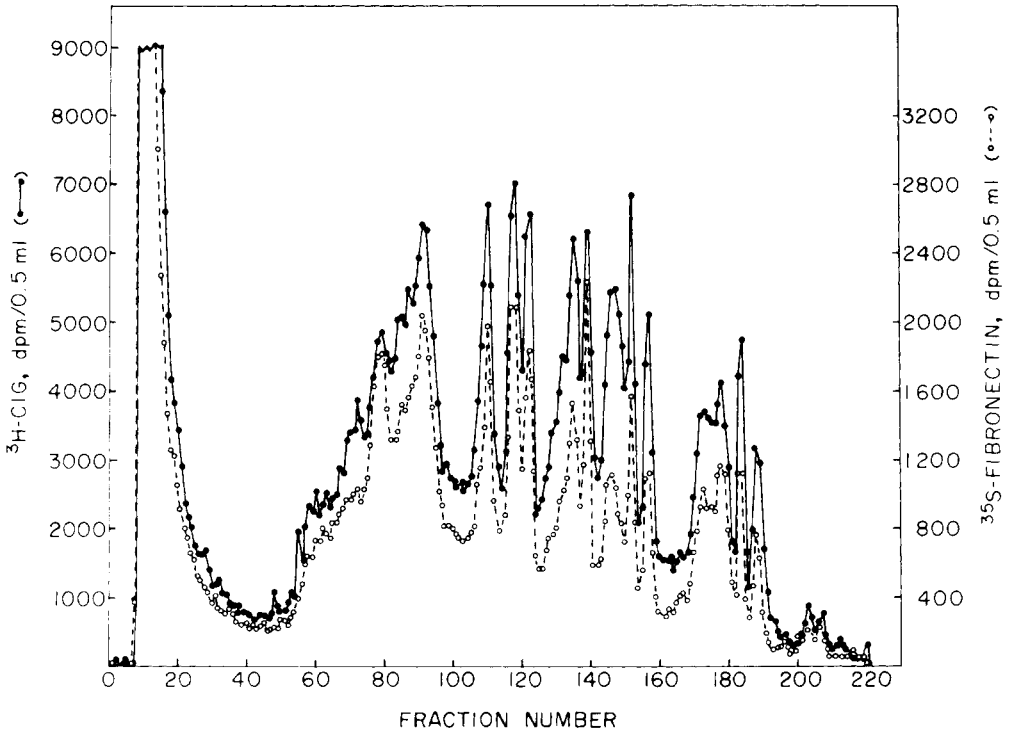

Fig. 2. Cation exchange chromatography of trypsin digests of CIG and fibronectin. [^{35}S] cysteine-labeled fibronectin was purified from amniotic fluid cell culture medium by precipitation with anti-CIG antibody and was reduced and carboxymethylated with iodoacetic acid. CIG was alkylated similarly but using [^{3}H] iodoacetic acid. The preparations of fibronectin and CIG were mixed and cleaved with trypsin, and the resulting peptides were separated on a UR-30 (Beckman) column using a multistep pyridine acetate gradient [27].

1 and 2). The fibronectin band is more diffuse and the average apparent molecular weight of the component chain after reduction of disulfide bonds is 235,000, in contrast to a calculated molecular weight of 220,000 for CIG. The peptides resulting from limited cleavage of CIG and fibronectin with cathepsin D and mast cell protease are also shown in Figure 3. In many instances the peptides derived from fibronectin are more heterogeneous

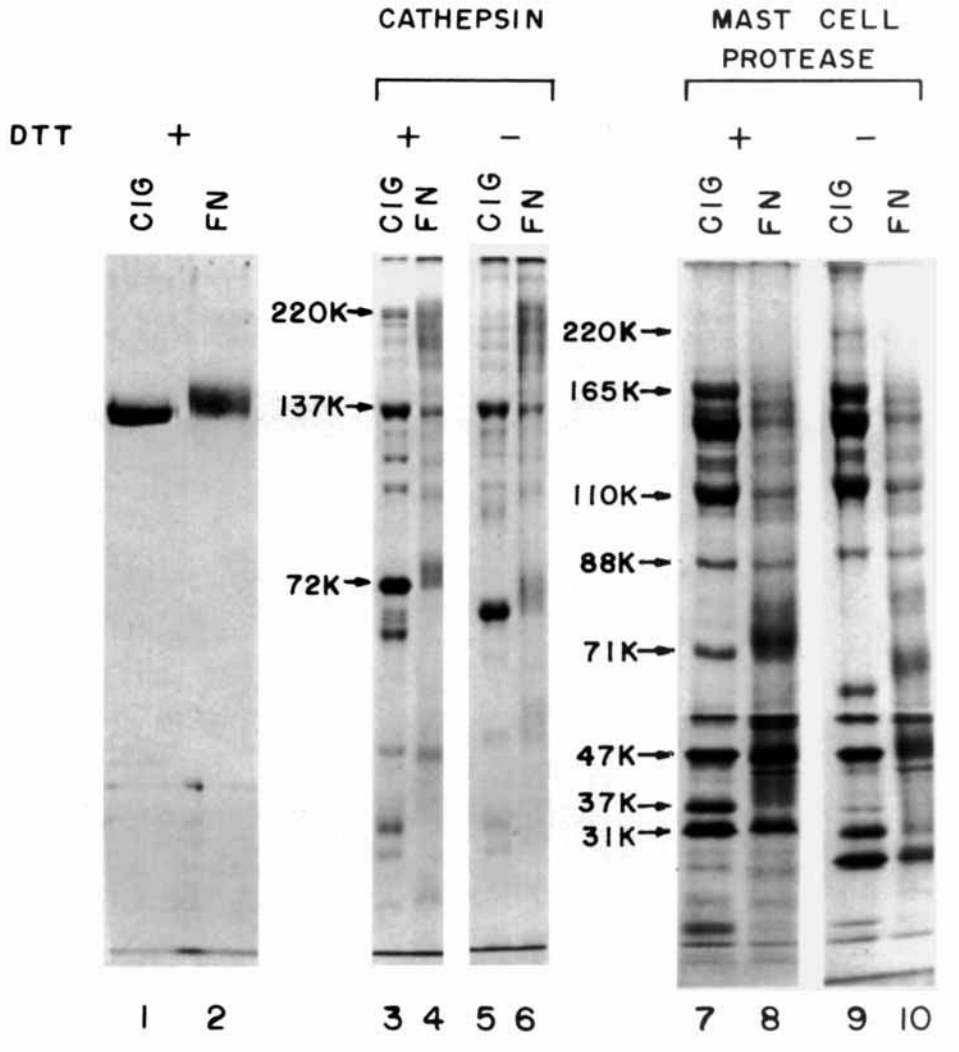

Fig. 3. SDS-polyacrylamide gel electrophoresis of CIG, fibronectin (FN), and their enzymatically derived fragments. Lanes 1, 2: CIG and fibronectin, 5 μg each. Lanes 3–6: Fibronectin and CIG treated with cathepsin D; 20 μg of protein was added to each lane. Lane 7–10: Fibronectin and CIG treated with mast cell protease; 30 μg CIG protein and 40 μg of fibronectin protein was added to each lane. Electrophoresis was performed in the absence (-) or presence (+) of 50 mM DTT on single-layer or composite slab gels. Lanes 1, 2: 5% acrylamide with 10% base; lanes 3–6, 7.5% acrylamide; lane 7–10, 7.5% acrylamide with 12.5% base. Gels were stained with Coomassie blue. The molecular weights indicated by the arrows were obtained by comparison with a mixture of globular proteins of known molecular weights and apply only to lanes 3 and 4 and to lanes 7 and 8.

TABLE II. Carbohydrate Composition of Fibronectin and Cold-Insoluble Globulin Expressed as Percentage Dry Weight

	Amniotic fluid fibronectin	Plasma CIG
Fucose	0.4	Trace
Mannose	1.1	1.0
Galactose	1.7	1.0
N-acetylglucosamine	2.5	1.8
N-acetylgalactosamine	0.7	0.1
Sialic acid	0.6	0.7
Total	7.0	4.6

*Several preparations of each protein were used; the values are an average of at least three determinations.

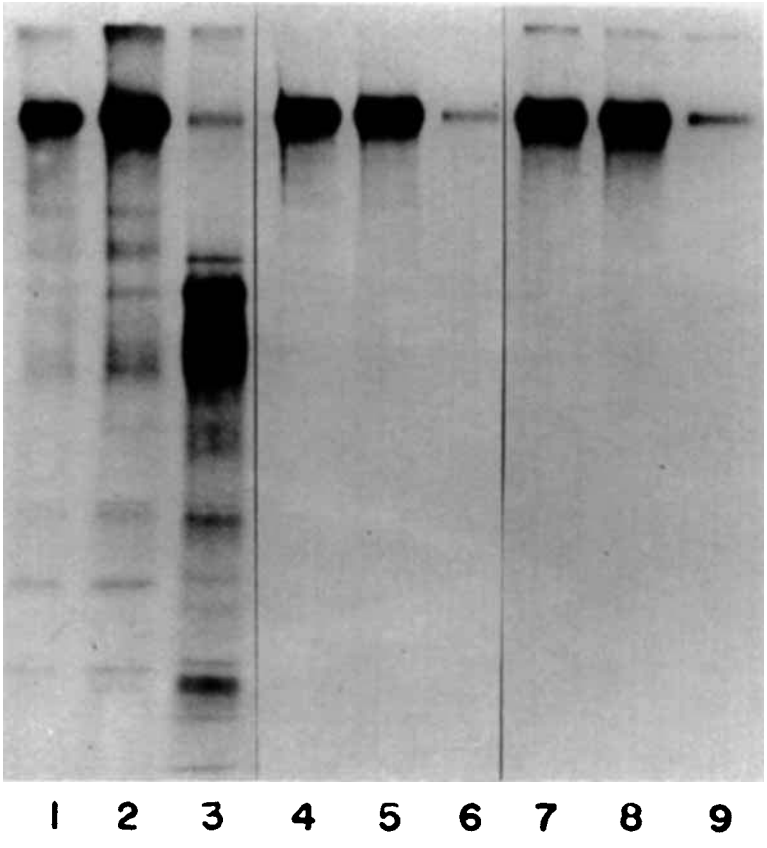

Fig. 4. SDS polyacrylamide gel electrophoresis of amniotic fluid cell fractions. Slab gels were composed of 7% acrylamide with a 10% base and were prepared for autoradiography. Lanes 1–3: [^{3}H] mannose-labeled cells precipitated with trichloroacetic acid; lane 1: culture medium; lane 2: urea extract; lane 3: cell homogenate. Lanes 4–6: [^{3}H] mannose-labeled cells precipitated with anti-fibronectin antibodies; lane 4: culture medium; lane 5: urea extract; lane 6: cell homogenate. Lanes 7–9: [^{3}H] glucosamine-labeled cells precipitated with antifibronectin antibodies; lane 7: culture medium; lane 8: urea extract; lane 9: cell homogenate.

and migrate more slowly than the corresponding peptides from CIG. This behavior is particularly prominent in the case of a 72,000 dalton fragment produced by cathepsin D and the effect is seen whether the gels are run in the absence or presence of a reducing agent (Fig. 3, lanes 3–6). Similar observations have been made using radiolabeled fibronectin from cell culture medium [18]. In addition there are other differences in the cleavage patterns obtained with the two proteases. Notably, at least two low-molecular-weight peptides in digests of CIG do not have prominent counterparts in digests of fibronectin (Fig. 3, compare lanes 3 and 4 and 9 and 10). These results have been reproduced with three different preparations of the two proteins.

Recent experiments have suggested that the electrophoretic heterogeneity of fibronectin can be accounted for by variable glycosylation of the protein [4]. The extent of glycosylation of CIG and fibronectin was therefore compared (Table II). Several differences were observed. Whereas amniotic fluid fibronectin contains significant amounts of fucose and N-acetylgalactosamine, these residues were present in only trace or very low amounts in CIG. Fibronectin also contained increased amounts of galactose and N-acetylglucosamine which contribute to a higher total carbohydrate content of fibronectin.

The polydispersity of fibronectin was further investigated using the glycoprotein isolated from different fractions of cells in culture and by comparing the size of the oligosaccharide-containing peptides. Fibronectin secreted by amniotic fluid cells into culture medium, extracted from cell surfaces with urea or obtained by detergent homogenization of of cell layers, was compared (Fig. 4). A high proportion of the radiolabeled glycoproteins secreted by these cells could be accounted for as fibronectin in the culture medium (Fig. 4, lanes 1, 4, and 7) and in the urea-soluble fractions (Fig. 4, lanes 2, 5, and 8). However, fibronectin constitutes only a small proportion of the total radioactive proteins obtained from the cell layer (Fig. 4, lane 3). Fibronectin could be purified from detergent homogenates of cells by immune precipitation (Fig. 4, lanes 6 and 9).

The oligosaccharide-containing peptides of soluble, immune-precipitated fibronectin were obtained by extensive cleavage of the polypeptide with pronase; the elution pattern of these glycopeptides is shown in Figure 5. The majority of the mannose and glucosamine migrates as a single peak (Fig. 5–1), in agreement with previous reports [30]. The majority of the carbohydrate in fibronectin is therefore in chains of uniform or nearly uniform length. In contrast, carbohydrate from cell layer-derived fibronectin preparations appeared as two major peaks (Fig. 5–2). One peak eluting in the void volume was a glucosamine-rich component containing essentially no mannose; a second peak, containing both glucosamine and mannose, eluted in the same position as the glycopeptides from soluble fibronectin. The glucosamine-rich component is most likely a pronase-resistant core derived from proteoglycan that was coprecipitated with fibronectin by antibodies. Much less proteoglycan was found to precipitate with medium or cell surface fibronectin, as indicated in Figure 5–1.

DISCUSSION

Fibronectin and CIG cross-react immunologically and are indistinguishable by cross-inhibition assays. Chemical and enzymatic cleavage of the two proteins also show extensive structural similarities. Reports from several sources suggest that fibronectin and CIG also share many of their biologic properties. However, as noted earlier, fibronectin is more active in mediating cell attachment [21] and several times more effective in hemagglutination of glutaraldehyde-fixed sheep erythrocytes [24], and in restoring a

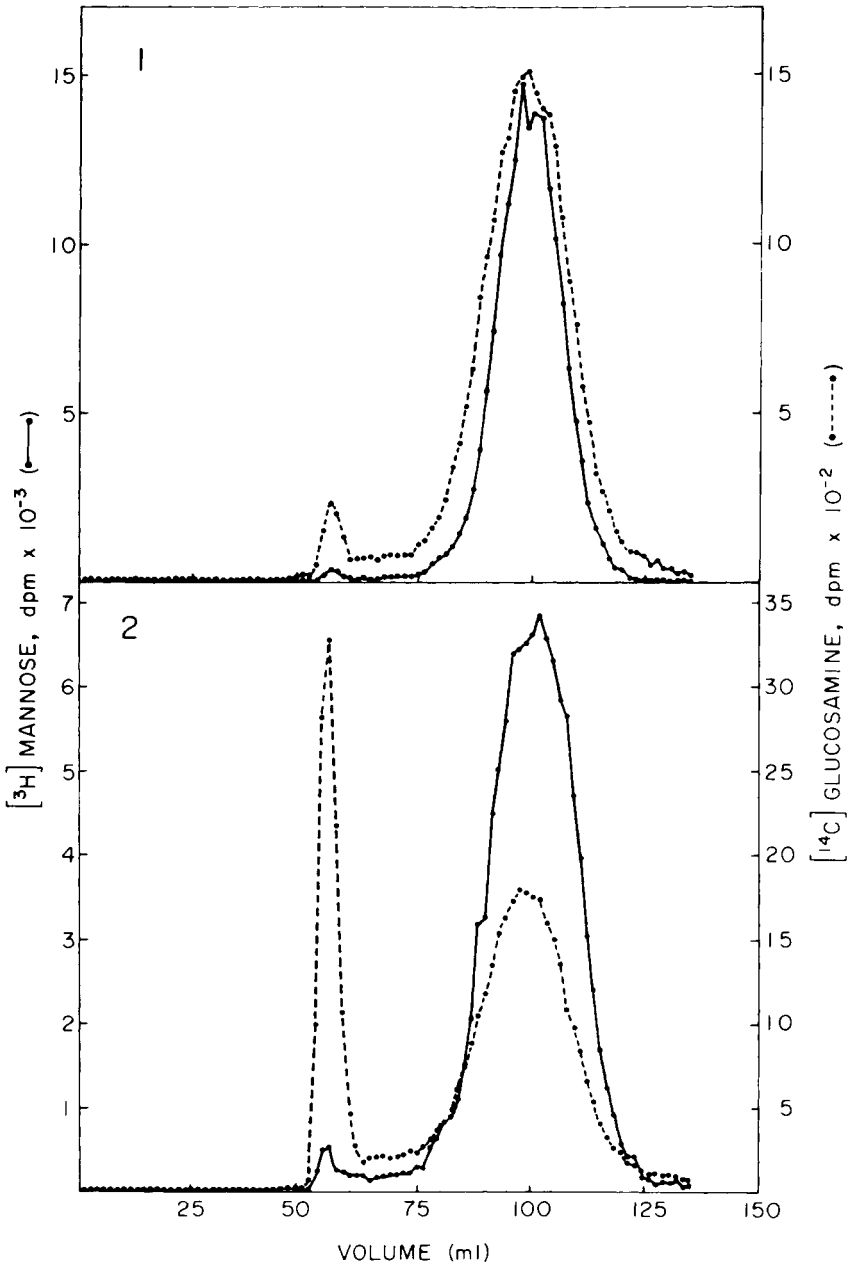

Fig. 5. Sephadex G-50 chromatography. Amniotic fluid cells were labeled with [^{3}H] mannose and [^{14}C] glucosamine, and culture medium, urea extracts, or cell homogenates were immune-precipitated separately. Mannose- and glucosamine-labeled fractions were mixed and digested with pronase before chromatography. Panel 1: Urea extracts. Panel 2: cell homogenates. The elution pattern of pronase digests of culture medium was the same as that shown in panel 1.

more normal morphology to transformed cells [24]. Recent reports have shown that when fibronectin is synthesized in the presence of tunicamycin, an inhibitor of protein glycosylation, the biological activity of the protein is retained [31]. This indicates that the carbohydrate moiety in fibronectin is not an essential requirement for some of the cell surface properties attributed to the glycoprotein. However, the nonglycosylated protein was shown to be twice as sensitive as the normal protein to proteolysis, both in vitro and using intact cells [31, 32]. These findings suggest that the increased levels of glycosylation of fibronectin could function to protect the protein from premature proteolysis from the cell surface. Differences in peptide pattern obtained after limited enzymatic cleavage of fibronectin and CIG indicate a difference in susceptibility of these two proteins to enzymatic cleavage. Differences in conformation may be responsible for these observations, since additional carbohydrate residues in fibronectin may limit cleavage at specific sites by these proteases. Additional differences in apoprotein structure between fibronectin and CIG, however, cannot be excluded.

A limited amount of proteoglycan may copurify with fibronectin, and although chromatography in SDS-containing buffers would be expected to disrupt the interaction of fibronectin with carbohydrate-containing contaminants, it is difficult to completely eliminate this possibility. Recently a high-molecular-weight, base-stable heteroglycan associated with isolated cell surface fibronectin was described [30]. This oligosaccharide contained galactose, N-acetylglucosamine, and N-acetylgalactosamine as major sugars.

A variety of cell types grown in culture have the ability to synthesize fibronectin or a fibronectin-like molecule that appears as a polydisperse band on SDS gels. The differences that exist between fibronectin and CIG, and the location of CIG in the circulatory system, suggest that the product synthesized and secreted by such cells in tissues could be a precursor to fibronectin found in plasma. Partial deglycosylation could account for the change in migration of fibronectin from a polydisperse band on SDS gels to a doublet represented by the chains of CIG. In this regard, the increased carbohydrate content and slower electrophoretic migration of amniotic fluid fibronectin cannot be attributed to its fetal origin, since human fetal and adult CIG migrate identically on SDS gels (unpublished observations).

Alternatively, CIG may be a synthetic product of cells which are designed to function as a source of plasma proteins, such as hepatocytes or cells associated with the vascular system. This raises the possibility that, depending on their requirements, different cell types may produce different forms of fibronectin. A number of observations have now been made which support the notion that fibronectin is not required by all cells in mediating the interaction of cells with their environment [33]; indeed in the case of epidermal cells and hepatocytes fibronectin does not appear to be required for attachment to a collagenous substratum [34, 35].

Our studies indicate that fibronectin is associated with a pronase-resistant high-molecular-weight glycan, presumably proteoglycan, in amniotic fluid cell layers and that the complex can be precipitated by antibodies to fibronectin. Earlier reports indicate that CIG binds to heparin [36] and that fibronectin is associated with glycosaminoglycan in NIL hamster fibroblasts [37]. We have used amniotic fluid cells to study the incorporation of Na_2 [$^{35}SO_4$]; the uptake of this isotope into a major component, presumably proteoglycan, which is secreted into the culture medium was shown (G. Balian, E. M. Click, and P. Bornstein, unpublished observation). Both fibronectin and proteoglycan are therefore likely to be present in the culture medium of cells but coprecipitation occurs only with homogenates of whole cells. The association of fibronectin and proteoglycan may

not occur in culture medium (ie, in solution) or in urea-extractable material because a fibrillar matrix may be required for the alignment of fibronectin and subsequent interaction with proteoglycan.

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