Human placental fibronectin was isolated from fresh term placenta by urea extraction and purified by gelatin affinity chromatography. A 44-kDa chymotryptic fragment, also purified by gelatin affinity chromatography, gave a broad, diffuse band on polyacrylamide gel electrophoresis, whereas the analogous 43-kDa fragment from human plasma fibronectin migrated as a defined, narrow band. Upon extended treatment with endo-β-galactosidase from Escherichia freundii, the 44-kDa chymotryptic gelatin-binding fragment from placental fibronectin changed its behavior on gel electrophoresis and migrated as a narrower, more defined band. The carbohydrates on human placental fibronectin contained a large percentage of polylactosamine carbohydrate as plasma fibronectin. NH2-terminal amino acid sequence analysis of the chymotryptic gelatin-binding fragments from both fibronectins showed the first 21 residues to be identical. Tryptic and chymotryptic peptide maps of the gelatin-binding fragment from placental fibronectin, however, showed differences including several protease-resistant domains not found in the analogous fragment from plasma fibronectin.

Intact placental fibronectin contains 20,000 Da of carbohydrate, whereas plasma fibronectin contains 11,000 Da. Placental fibronectin is more protease-resistant than plasma fibronectin, possibly due to the additional carbohydrate. Polyclonal antibodies against either fibronectin completely cross-react with amnionic fluid fibronectin, placental fibronectin, and plasma fibronectin upon Ouchterlony immunodiffusion. Human fibronectins of putatively the same polypeptide structure are, therefore, glycosylated in a dramatically different fashion, depending on the tissue of expression. If the patterns of glycosylation comprise the only difference in the glycoprotein, this may confer the characteristic protease resistance found for each of the fibronectins.

Fibronectins are a group of multifunctional cell surface and secreted glycoproteins of subunit Mr = 200,000 to 250,000, originally found on mammalian and avian fibroblasts, and later shown to be related to cold insoluble globulin in plasma. An extensive literature on this subject has been recently reviewed by Yamada (2) and needs not be reiterated here.

Fibronectins from tissue and plasma in several species possess differing chemical, physical, and biological properties. In avian fibronectins, a cellular form of fibronectin isolated from chick embryo fibroblasts is less soluble than the plasma form, and has greater properties of promoting cell spreading in tissue culture. Fibronectins also express disparate carbohydrates according to species, although the major glycopeptide from human (4) and hamster (5) plasma fibronectins has an almost identical structure. It has been reported that hamster fibronectin glycopeptides from plasma and cellular sources differ only in the presence of fucose and a diminution of sialic acid on the cellular form. Bovine plasma fibronectin has an unusual N-linked carbohydrate with a branched glucosamine containing a sialic acid (6).

Human fibronectins have been studied from various sources, including plasma (4, 7-16), amniotic fluid (17, 18), placenta (1, 19), and fibroblasts grown in culture (20, 21).

pFn1 structure has been extensively studied (22-28), has been partially sequenced (29, 30), and has had its major carbohydrate characterized (4, 18). It contains about 5% carbohydrate in the form of partially desialylated N-linked biantennary structures (4), with a small amount of larger oligosaccharide present (4). aFn1 has a similar structure, but has been reported to contain 9% carbohydrate with increased galactose and glucosamine (18, 31, 32). cFnl has been isolated, located in the tissue by immunofluorescence, and studied by Bray (33) and others (19).

In this report, we will show that cFn1 contains appreciable amounts of an unusual polylactosamine structure, and a much higher carbohydrate content than pFn1, amounting to 9% of

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1 The abbreviations used are: pFn1, human adult plasma fibronectin; cFn1, human placental cellular fibronectin; aFn1, human amniotic fluid fibronectin; TPCK-treated trypsin, l-tosylamido-2-phenoxyethylchloromethyl ketone-treated trypsin; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; HPLC, high performance liquid chromatography; ConA, concanavalin A.
EXPERIMENTAL PROCEDURES

Materials—Chymotrypsin (49 units/mg) was obtained from Worthington Biochemical Corporation. TPCK-treated trypsin, gelatin (from swine skin, type I), fetuin (type III), transferrin (human), soybean trypsin inhibitor, protease (type XIV), and PMSF were obtained from Sigma Chemical Company. ConA-Sepharose 4B, Sepharose 6B, and Sepharose G-10, G-50, and G-100 were obtained from Pharmacia (Uppsala, Sweden). Bio-Gel P-10 was purchased from Bio-Rad Laboratories. Endo-β-galactosidase (from Escherichia freundii) was a generous gift from Drs. Y. T. Li and S. C. Li, Tulane University Medical Center, New Orleans, LA, and the Delta Regional Primate Research Center, Covington, LA. Geissinulin-Sepharose 4B was prepared according to Porath and Kristiansen (34). Other reagents used were of analytical grade and obtained from commercial suppliers.

Preparation of Fibronectin—pFn1 was purified from citrate-treated human plasma by gelatin-affinity chromatography according to the method of Hopper et al. (56) as modified by Enzell and Rousslahi (36). We used chromatography on Sepharose 6B in phosphate-buffered saline, pH 7.0, containing 4 M urea as an additional step after gelatin-Sepharose to ensure that fibronectin was free of plasma proteins, especially fibrinogen, which tends to co-purify with fibronectin in the cold (37).

cFn1 was extracted from fresh term placenta with 2 M urea in pH 7.0 phosphate-buffered saline, according to a modified method of Yamada and Weston (38). Normal placentas were collected immediately following delivery and washed extensively with cold running water through the hysterectomy cord. During the procedure, the placentas were maintained at 4 °C. After washing out the blood, the tissue was dissected, being careful to select areas of healthy, non-neoplastic tissue, and terminal villi were freed of any remaining decidua. The tissue was blended for 1 min after adding urea solution (2 M final concentration including the placental tissue as a solution) in phosphate-buffered saline, pH 7.0, and PMSF. The slurry was shaken overnight at 4 °C and centrifuged 20 min at 25,000 × g. The supernatant was diluted 20-fold or more to allow purification on a gelatin-Sepharose column. The isolated protein was analyzed by SDS-polyacrylamide gel electrophoresis after Sepharose 6B gel filtration.

As a note for the isolation of placental fibronectin, the tissue is washed extensively to remove all blood and decidua, until the white trophoblastic trees of the placenta remain. We obtain the highest yields of fibronectin using Yamada and Weston's (38) urea extraction procedure by adding enough urea to make the final concentration 2 M including the volume of placental tissue solution of urea, extraction procedure by Bray et al. (39), too was expensive for routine extraction. The yield is extremely variable and we do not entirely understand the parameters of variance. The best yields we have obtained are 130 mg of cFn1 from a single fresh placenta, extracted twice with urea. As little as 5 to 10 mg are sometimes obtained with fresh placentas, and we have not yet determined whether this is due to age of the fetus, duration of labor, time between birth and processing, or some genetic variation. Good yields are obtained from some placentas kept in ice directly after birth. Average yield is 20 to 50 mg/placenta.

Digestion of Plasma and Placental Cellular Fibronectin with Chymotrypsin and Separation of Gelatin-Binding Fragments—After passing through a Sephacryl G-10 column to remove urea, pFn1 and cFn1 (1 mg/ml) were digested by chymotrypsin in pH 7.0, 0.05 M Tris-HCl buffer containing 0.15 M NaCl and 0.3 M CaCl2 at an enzyme/substrate ratio of 1:100 in a volume of 4 ml. The reaction was terminated by adding 2 mM PMSF and soybean trypsin inhibitor. As a control, no enzyme was added.

Preparation and Fractionation of Glycopeptides from pFn1, cFn1 and Their Chymotryptic Fragments—The glycopeptides of cFn1 were released by alkaline borohydride treatment (1 M NaOH, 4 M NaH3PO4, 80 °C, 48 h) and the chymotrypsin fragments of pFn1 and cFn1 were digested with pronase. The solubilized glycopeptides were isolated by chromatography on a 30 × 15 cm Sephadex G-50 column eluted with 0.1 M pyridine acetate buffer, pH 5.0 or a 90 × 1.5 cm Bio-Gel P-6 column eluted with 15% acetic acid. Fractions were collected and assayed by the phenol-sulfuric acid method (40), using glucose as the standard. These glycopeptides were further fractionated on ConA-Sepharose 4B (41).

Endo-β-galactosidase Digestion of the Glycopeptides Released from Chymotryptic Fragments of cFn1—1.5 μmol of glycopeptides were digested with endo-β-galactosidase in 0.1 M sodium acetate buffer, pH 6.8, 37 °C for 24 h and sized on a Bio-Gel P-6 gel permeation chromatography column.

Determination of Carbohydrate Composition and Methylation Analysis of Glycopeptides—The carbohydrate compositions of pFn1, cFn1, and their chymotryptic fragments were determined after methanolysis by gas-liquid chromatography of the trimethylsilylated methyl glycosides (42) on a Shimadzu Mini-1 gas chromatograph, equipped with a flame ionization detector and a column (6 ft × 0.025 in) of 5% OV-101 on 100/120 Supelcoport (Supelco Inc.); carrier gas was nitrogen at 40 ml/min. Peaks were integrated using a Spectra Physics SP-4100 computing integrator. Linkage positions were determined by methylation analysis (43, 44).

Polyacrylamide Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was carried out according to Laemmli (45) with 4% stacking and 7.5% or 12% resolving gels. Protein samples were prepared by heating at 100 °C for 2 min in pH 6.5, 0.05 M Tris-HCl, containing 6% sucrose, 1% SDS, and 0.001% bromophenol blue, with or without the addition of (0.1 M dithiothreitol). The apparent molecular weight was estimated by using the following proteins as standards: myosin, 205,000; β-galactosidase, 116,000; phosphorylase b, 94,000; bovine serum albumin, 68,000; ovalbumin, 43,000; carbonic anhydrase, 28,000; trypsinogen, PMSF-treated, 24,000; β-lactoglobulin, 18,400; and lysozyme, 14,300. The gels were stained for proteins with Coomassie blue or with periodic acid-Schiff stain for carbohydrate.

Amino Acid Analysis—The amino acid compositions of pFn1, cFn1, the 43-kDa fragment from pFn1, and the 44-kDa fragment from cFn1 were determined on a Beckman 121 MB analyzer according to Del Valle and Shively (46). Samples were hydrolyzed in evacuated tubes for 24 h at 110 °C with 6 M HCl containing 0.02% β-mercaptoethanol.

Microsequence Analysis—One to 4 nmol of S-P1-Carboxymethylated (47) 43-kDa pFn1 fragment and cFn1 44-kDa fragment were analyzed on either a modified spinning cup (Beckman 890 C Sequencer (48)) or on a City of Hope gel phase sequencer. Amino acid phenylthiohydantoin (PthNCS) derivatives were separated and identified by HPLC on a DuPont Zorbax ODS column installed in a Waters Associates chromatograph equipped with 254- and 313-nm detectors. Peaks were integrated and gradient elution was controlled by a Spectra Physics 4000 integrator system.

Preparation of Tryptic and Chymotryptic Digests of the Gelatin-binding Fragments 43-44-kDa Fragments—After reduction and S-alkylation with iodo[i-14C]acetic acid, the gelatin-binding fragments were digested with TPCK-treated trypsin and HPLC-purified chymotrypsin in 0.2 M NH4HCO3 (pH 8.0) at 37 °C for 24 h (chymotrypsin digestion) and 48 h (trypsin digestion) with an enzyme to protein ratio of 2:100 (w/w).

Reverse Phase HPLC—Reverse phase HPLC consisted of a high pressure Milton Roy minipump with a Rheodyne sample injector (sample loop of 1.0 ml). The UV detection system included an LKB 11300 gradient mixer, and a LKB 2135 Ultracon-S. Chromatography was performed on a Vydac C4 column, and effluent was monitored for absorbance at 206 nm. Elution of the peptides was achieved by using a linear gradient from solvent A (0.10% trifluoroacetic acid, pH 2.1) to solvent B (0.10% trifluoroacetic acid, 90% CH3CN) over a period of 1.5 h at a constant flow rate of 44 ml/h. The buffers were continuously degassed by flushing with helium. The details have previously been described (29).

RESULTS

Chymotryptic Gelatin-binding Fragments from cFn1 and pFn1—Fig. 1 shows a polyacrylamide gel electrophoretogram of affinity purified plasma fibronectin in lane 2 and placental fibronectin in lane 6. The chymotryptic gelatin-binding fragment from pFn1, estimated at 43 kDa, is in lane 4 and its counterpart from placental fibronectin (cFn1) is the diffuse band in lane 7. Fig. 2 shows the electrophoretic behavior of the cFn1 gelatin-binding fragment after extensive digestion with endo-β-galactosidase, as compared with control, suggesting that the presence of polyglactosamine chains is responsible for the original diffuse appearance of the 44-kDa fragment.
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Amino Acid Analysis—The amino acid compositions of intact plasma and placental fibronectins are shown in Table I. The compositions are closely similar and reflect a high degree of structural relatedness. The compositions of the chymotryptic, gelatin-binding fragments are identical within experimental error. Each fragment contained 24 cysteine residues and a substantial amount of glucosamine (not shown).

NH$_2$-Terminal Amino Acid Sequence of the 43–44-kDa Chymotryptic Gelatin-binding Fragments—Table II gives the NH$_2$-terminal amino acid sequence of the gelatin-binding chymotryptic fragment from both pFnl (Sequence 1) and cFnl (Sequence 2), as compared to a sequence beginning with amino acid residue 264 in bovine fibronectin (Sequence 4) reported by Petersen et al. (49). The NH$_2$-terminal sequence for intact human plasma fibronectin has been shown by two laboratories to be Glu-Ala-Glu-Glu-, the same as for bovine plasma fibronectin. Approximately 0.8 nmol of the plasma pFnl 43-kDa chymotryptic fragment was directly sequenced without prior S-carboxymethylation. A single sequence with a good signal to noise ratio (Sequence 3) was obtained through 21 cycles of Edman degradation. This sequence was a subsequence of that previously reported (29) and was not examined further.

Approximately 2.0 nmol of cFnl 44-kDa chymotryptic fragment was sequenced through 30 residues (Sequence 2) with a clear identification of a cysteine at cycle 14. The sequence was identical with the pFnl (Sequence 3) through the first 21 residues, and identical with a sequence in the bovine plasma fibronectin gelatin-binding region (Sequence 4, residues 264–289). Only 2 residues of the 30 differ between bovine and human in the region, demonstrating a highly conserved amino acid sequence. There is, as yet, no evidence of amino acid substitutions between the plasma and placental forms of human fibronectin. The 29-kDa gelatin-binding tryptic fragment (29) corresponds to the NH$_2$-terminal regions of the 43–44-kDa chymotryptic fragments reported here. Thus, it can be concluded that only the 29-kDa NH$_2$-terminal portion of the larger fragments is required to bind collagen.

Peptide Mapping—Tryptic peptide maps using reverse phase high performance liquid chromatography, prepared after 48 h of trypsin digestion of the 43–44-kDa chymotryptic fragments from pFnl and cFnl, are shown in Fig. 3. Trypsin digestion of the plasma chymotryptic fragment reaches a limit in 24 h, but the placental fragment requires at least 48 h. At the end of 48 h, the two maps look considerably different, with the placental fragment yielding mostly larger fragments.
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**NH2-terminal amino acid sequences**

| Human fibronectin gelatin-binding chymotryptic fragments |  |
|----------------------------------------------------------|--|
| 1. Human adult plasma (pFn1)†                         | Chymotrypsin  |
| JQPQHPQPPPYGHCVTDSGVV                                     |  |
| 2. Human term placenta (cFn1)§                          | Chymotrypsin  |
| JQPQHPQPPPYGHCVTDSGVVY SVGM QWLK                       |  |
| Human fibronectin gelatin-binding tryptic fragment      |  |
| 3. Human adult plasma (pFn1)§                            | Trypsin  |
| JAAVYPQPPPYGHCVTDSGVVY                                   |  |
| 4. Bovine adult plasma†                                   | Plasmin  |
| ITAIYQPQPPPYGHCVTDSGVVY                                  |  |

Bovine fibronectin, subsequence, residues 260–289 from NH2 terminus

| Amino acid | 260 | 264 | 270 | 250 | 290 |
|------------|-----|-----|-----|-----|-----|
| 100%       | 0   | 0   | 0   | 0   | 0   |
| 0%         | 0   | 0   | 0   | 0   | 0   |
| 20%        | 8.75| 8.75| 0   | 0   | 0   |
| 40%        | 17.5| 17.5| 17.5| 0   | 0   |
| 60%        | 26.25| 26.25| 26.25| 26.25| 0   |
| 80%        | 35| 35| 35| 35| 0   |

†This report.
§Ref. 29.
§Ref. 49.

![Fig. 3. Tryptic peptide map of chymotryptic gelatin-binding fragments from human plasma fibronectin (pFn1) and human placental fibronectin (cFn1). At the top, 5 nmol of the 44-kDa cFn1 chymotryptic fragment were digested with 2% trypsin for 48 h. Both samples were denatured, reduced, and S-carboxymethylated prior to trypsinnization. The peptides were fractionated on a VYDAC C18 column using a gradient from 100% solvent A (0.10% aqueous trifluoroacetic acid) to 70% solvent B (0.1% trifluoroacetic acid/McCN) over 90 min at a flow rate of 0.8 ml/min. Only the first 60 min of the run are shown since no peak eluted beyond this point in the gradient. From this result, it is concluded that the placental fragment has higher resistance to trypsin cleavage even after S-carboxymethylation. In comparison to this result, chymotrypsin cleavage is complete for both fragments in 24 h and yields more similar maps, as shown in Fig. 4. The largest chymotryptic peak (39 min) for the placental cFn1 fragment (Fig. 4) and the peak preceding it (38 min) each contained cysteine and had similar amino acid compositions for the corresponding peaks obtained for the pFn1 and cFn1 fragments. Each peak contained several components. The exception was that glucosamine was detected in the much larger 38-min peak for cFn1, but not for pFn1, suggesting the presence of a saccharide-containing chymotrypsin-resistant region in cFn1 that is not glycosylated in pFn1.](http://www.jbc.org/)

**Fig. 4. Peptide map after extensive chymotryptic digestion of the mild chymotryptic gelatin-binding fragments from human plasma fibronectin pFn1 (top), and human placental fibronectin cFn1 (bottom). The conditions are similar to those in Fig. 3, except that a sample of 5 nmol of each peptide was digested with 2% chymotrypsin for 24 h.**
The 44-kDa fragment from cFn1 contains some highly trypsin-resistant regions in the time zones 35 to 40, 45 to 50, and 50 to 60 min (Fig. 3) when compared with similar regions in the tryptic map from pFn1. These are presumably due to glycosylation differences, although they may reflect amino acid substitutions. Extensive further work will need to be performed to determine this difference. These peptide maps also contain a large number of corresponding peaks.

**Immunological Identity of Human Fibronectins**—Fig. 5 shows Ouchterlony immunodiffusion with polyclonal antibodies prepared in rabbits against pFn1 and cFn1, displaying complete cross-reactivity among pFn1, cFn1, and aFn1.

**Saccharides from pFn1 and cFn1**—Pronase digests of the gelatin-binding and nonbinding domains of pFn1 and cFn1 yielded glycopeptides which were fractionated by concanavalin A-Sepharose affinity chromatography and by Bio-Gel P-6 gel permeation chromatography. Fig. 6 shows the flow chart for the preparations of the fractions which were isolated. First we will consider the glycopeptides obtained from the gelatin-binding 43-44-kDa chymotryptic fragments, illustrated on the right side of the flow chart.

**Carbohydrates of the 43–44 kDa Chymotryptic Gelatin-binding Fragments**—Tables III and IV show the sugar composition of the glycopeptides obtained after pronase digestion of the 43–44 kDa gelatin-binding chymotryptic fragments from pFn1 and cFn1, respectively. Values for ConA-weakly bound (columns 1 and 3) and nonbinding (columns 2 and 4) glycopeptides are shown. The ConA-unbound fractions from human plasma fibronectin (Table III, columns 2 and 4) are only a small fraction of the glycopeptides, as was also reported previously (4). The fraction that does not bind to ConA contains 5 mol of galactose/3 mol of mannose and thus may contain poly lactosamine sequences. In Table III (column 1), the data indicate that only one oligosaccharide is present on the 43-kDa gelatin-binding fragment from pFn1, which is of the biantennary type, lacking some sialylation.

![Fig. 5. Immunodiffusion in agarose. Well A, rabbit anti-human plasma fibronectin; well E, rabbit anti-placental fibronectin; wells B and F, human plasma fibronectin; wells C and H, human amniotic fluid fibronectin; wells D and G, human placental fibronectin.](http://www.jbc.org/)

**Fig. 6. Flow chart for preparation of chymotryptic fractions of human fibronectins from plasma and placenta, and for isolation of fractions of glycopeptides from the chymotryptic fragments.** Affinity columns indicated are gelatin-Sepharose for the peptides and concanavalin A-Sepharose for the glycopeptides. Fractions A–H were chromatographed on Bio-Rad P-6 gel permeation resin as shown in Fig. 7.

**TABLE III**

| Sugar | Gelatin-binding 43-kDa ConA binding | Non-gelatin binding 180-kDa ConA binding |
|-------|-------------------------------------|------------------------------------------|
|       | mol                                 | mol                                      |
| Mannose | 2.5                                  | 0.1                                     | 11.3 | 0.86 |
| Galactose | 2.08                                 | <0.1                                   | 6.01 | 1.49 |
| GlcNAc | 2.36                                 | 0.0                                     | 4.05 | 1.04 |
| NANAa | 0.67                                 | <0.1                                    | 1.93 | 0.72 |
| Fucose | 0.00                                 | 0.0                                     | 0.27 | 0.21 |
| Total | 7.61                                 | 0.1                                     | 23.56 | 4.32 |

*a Sum of all remaining non-gelatin-binding fragments.

b NANA, N-acetylleucaminic acid.

On the other hand, in Table IV (columns 1 and 2), composition data for the 44-kDa gelatin-binding fragment from cFn1 show the presence of almost 6 mol of mannose on two different kinds of glycopeptides, indicating the presence of two N-linked saccharide chains on this analogous fragment. Moreover, the composition data indicate that the ½ of this fraction that does not bind to ConA (column 2) has high galactose and glucosamine, a poly lactosamine characteristic. Enzymatic digestion of this fraction results in partial degradation of components as shown below. Thus, the glycosylation of the gelatin-binding fragment from these two human fibronectins is different in two ways: (i) with respect to the number of chains, and (ii) the type of saccharide.

We noticed that the
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TABLE IV
Sugar composition of human placental (fetal) fibronectin: chymotryptic fragments

| Sugar       | Gelatin-binding 43-kDa ConA binding | Non-gelatin-binding 180-kDa ConA binding |
|-------------|--------------------------------------|-----------------------------------------|
|             | Yes | No | Yes | No |
| Mannose     | 2.57 | 3.14 | 3.8 | 2.6 |
| Galactose   | 2.24 | 6.24 | 2.9 | 11.5 |
| GlcNAc      | 2.9 | 8.07 | 3.1 | 9.7 |
| NANA*       | 1.03 | 1.02 | 0.84 | 2.24 |
| Fucose      | 0.35 | 1.08 | 0.43 | 2.3 |
| Total       | 9.09 | 19.06 | 11.07 | 28.3 |

* Sum of all remaining non-gelatin-binding fragments.

44-kDa fragment from cFn1 is more resistant to proteolysis than the 43-kDa fragment from pFn1. The strongest evidence for this was from peptide maps of the gelatin-binding fragments, on HPLC, as described above, revealing regions not degraded by trypsin in the 44-kDa cFn1 fragment that were not in the 43-kDa fragment from pFn1, as shown in Fig. 3.

Gel filtration chromatography on Bio-Gel P-6 of the fractions of glycopeptides obtained as shown in the flow chart in Fig. 6 are depicted in Fig. 7. The main portion of the glycopeptides from the 43-kDa fragment from pFn1 were weakly bound to concanavalin A-Sepharose (Fig. 7C) and only small amounts passed through the column (Fig. 7D). Of the glycopeptides which weakly bound to ConA, a small portion appeared as a larger molecular weight peak eluting earlier than a standard of transferrin glycopeptides (Fig. 7C). Methylation analysis of the major fraction which weakly bound to ConA gave derivatives which confirmed the transferrin-like biantennary structure (4).

More total carbohydrate was present on the 44-kDa chymotryptic fragment from cFn1 as shown in Fig. 7, G and H. A similar amount of biantennary structure, weakly binding to ConA, was found on the 44-kDa fragment, but a large quantity of heterogeneous glycopeptides passed through the ConA column, some of which appeared to be larger in size than the transferrin glycopeptide.

This amount of carbohydrate indicates that while perhaps one biantennary chain, on the average, is present on the pFn1 collagen-binding 43-kDa fragment, in contrast, two chains of N-linked carbohydrate are covalently linked to the 44-kDa fragment from cFn1. Fig. 8 shows the results of endo-β-galactosidase digestion of the broad mixture of glycopeptides which are shown in Fig. 7H. This fraction was degraded, in part, to lower molecular weight oligosaccharides, leaving a residue with the mobility of a biantennary structure. This was an indication of the presence of heterogeneous lengths of polyactosamine oligomers attached to the glycopeptide. This confirms the studies on the intact 44-kDa fragment from cFn1 as mentioned above, which show that the polypeptide migrates as a much narrower band upon treatment with endo-β-galactosidase.

Carbohydrates of Non-gelatin-binding Fragments from pFn1 and cFn1—The majority of the chymotryptic polypeptide fragments of pFn1 and cFn1 pass through the gelatin-Sepharose affinity column. This mixture of polypeptides was pronase digested, and the resulting glycopeptides chromatographed on concanavalin A-Sepharose and Bio-Gel P-6. This scheme is shown on the left side of Fig. 6. In pFn1, ⅔ of the glycopeptides bind weakly to ConA-Sepharose and the remainder pass through the column. The fraction weakly bound...
to ConA migrates with the transferrin biantennary glycopeptide and gives a characteristic methylation pattern for N-linked biantennary structures. The fraction from pFn1 not bound to ConA exhibits a major peak which elutes in the area of fetuin triantennary glycopeptides and a small amount of structures larger in molecular weight.

The carbohydrates on the gelatin-unbound cFnl which are not bound to ConA show striking differences from the plasma type of glycopeptide. As shown in Fig. 7E, a large proportion of these structures elute in a peak which has an average $M_r = 8,000$, earlier than the fetuin glycopeptide. This peak has considerable size distribution. Pool 1 from Fig. 7E was treated with endo-$\beta$-galactosidase, and appears to decrease in size to a $M_r = 3,000$ peak, as shown in Fig. 9, while releasing a series of oligolactosamine structures. Results of the methylation analysis of pool 1, Fig. 7E, are shown in Fig. 10. The presence of large amounts of 3-linked galactose (peak A) and 4-linked glucosamine (peak G) are a reflection of the polylactosamine nature of this pool as was also suggested above by its susceptibility to endo-$\beta$-galactosidase. The broad gel permeation distribution indicates a variable degree of polylactosamine polymer number. The presence of 2,4-linked mannose (peak C), 2,6-linked mannose (peak D), and 3,6-linked mannose (peak E), along with the absence of a significant peak for 2,6-linked mannose (peak B remains unidentified) indicates that the representative structure in this fraction is a tetra-antennary oligosaccharide of the complex type. Little galactose branching is evident as seen by the small 3,6-galactose peak (F).

The small terminal galactose peak preceding peak A indicates that most of the termini in this fraction are covered. No significant peak for terminal hexosamine was found. Since this fraction contains 2 sialic acids and 2 fucoses/3 mannoses, we suggest the structure shown in Fig. 11, with variable length antennae and since the apparent molecular weight range for this fraction is 4,000 to 15,000, the sum of the numbers $N_1-N_4$ in the chains. The numbers $N_1$ through $N_4$ are shown in Fig. 11 to indicate the variable nature of the polylactosamine-containing antennae and since the apparent molecular weight range for this fraction is 4,000 to 15,000, the sum of the numbers $N_1 + N_2 + N_3 + N_4$ ranges from 4 to 25. The lengths of the four antennae may well vary within one molecule. Methylation analysis of the smaller peaks released by endo-$\beta$-galactosidase treatment showed 3-linked galactose. Pool 2 from Fig. 7E has a mobility similar to fetuin glycopeptide and the methylation analysis confirmed this assignment, showing the following derivatives: 3-linked galactose, 2-linked mannose, and 3,6-linked and 2,4-linked mannose.

**DISCUSSION**

Yamada has reported that tunicamycin-treated chick fibroblasts produce fibronectin without carbohydrate which is much more susceptible to proteases (50). We have found that pFn1 and its 43-kDa gelatin-binding chymotryptic fragment are much more susceptible to proteases than cFnl, which may be due to the lower amount of carbohydrate on pFn1, although a difference in polypeptide sequence is not yet excluded. The cFnl 44-kDa gelatin-binding chymotryptic fragment is much more resistant to trypsin digestion than its counterpart from...
Human Placental Fibronectin

pFn1. If this protease resistance is due to the carbohydrate, which it appears to be, it will be of interest to determine the properties of the saccharides which confer the protease resistance. The possibilities would include the mere steric presence of more carbohydrate, the particular saccharide structures present, or the location on the polypeptide chain. Certain domains within the cFn1-44-kDa gelatin-binding fragment appear to be resistant to trypsin, while the amino acid composition for both gelatin-binding fragments from pFn1 and cFn1 are similar, the NH₂-terminal sequence is identical, and the immunological properties seem identical. Both gel behavior and protease resistance appear to be different in the two fibronectins, and this seems to be due to the presence of additional carbohydrate in the form of polylactosamine structures.

Polylactosamine oligomers attached to N-linked cores of glycoproteins were first found on band 3 protein of human erythrocyte membranes (51–53) and were later reported on permanent cell lines, including Chinese hamster ovary cells (54), K-562 cells (55–57), and GM-979 cells (58). Others later confirmed these findings (59–61). Several investigators, notably Feizi and her co-workers, and Muramatsu and his co-workers, have found immunological evidence for these structures on early mammalian embryos (62–69). cFn1, like band 3, is a cell surface protein. cFn1 apparently has a similar, if not identical, amino acid sequence as pFn1, a plasma form of the same protein. Since these proteins are immunologically indistinguishable, the major part of their chemical difference may lie in the carbohydrate. The biosynthetic source of pFn1 is probably hepatocytes (70) and, for cFn1, is probably placental fetal fibroblasts since these cells and adjoining basement membranes are coated with fibronectin as shown by immunofluorescence (32). Thus, somatic expression of putatively the same polypeptide in different tissues results in dramatically different carbohydrates being inserted on the polypeptide in the different cell types. This could be explained by the existence of more than one gene for fibronectin with slightly different amino acid sequence, which may determine the glycosylation sites and its extent. Another, more likely possibility is that different programs of glycosylation enzyme genes are expressed in various types of differentiated cells. The appearance of cFn1 on polyacrylamide gel electrophoresis as a diffuse band is carried over in the chymotryptic fragment which binds gelatin. Incubation of the protein or its chymotryptic gelatin-binding fragment with endo-β-galactosidase results in a narrow band on gel electrophoresis, similar to the effect of that enzyme on band 3 of human erythrocytes (71).

Apparently, no generality about carbohydrates on mammalian fibronectins can be made. In hamsters, it has been reported that all of the carbohydrate resides on a trypsic gelatin-binding fragment (5, 72) and that only the presence of fucose and diminution of sialic acid mark the difference between a cellular form of the enzyme and its plasma counterpart. In the human fibronectins, fucose is present on cFn1 to a greater extent than on pFn1, but it occurs in at least 1 or 2 mol of fucose/pFn1 molecule, located on the gelatin-unbound portion of pFn1 in the ConA-unbound fraction of glycopeptides. cFn1 contains 1 mol of fucose on the gelatin-bound fragment and 3 mol on the gelatin-unbound regions.

The carbohydrates on the two human fibronectins studied here are distributed much differently than on the hamster fibronectins. The human fibronectins have only a small part of the carbohydrates inserted near the gelatin-binding area and the majority is on the remainder of the molecule. However, the placental form seems to have two chains on this region versus one in the plasma form. The type of structures noted on bovine fibronectin (6) is different from both the hamster and human type. The previous reports of sugar composition on amniotic fluid fibronectin (31) with increased galactose and glucosamine seems to indicate that this form of the protein also may contain polylactosamine sequences. Whether this is only a property of certain fetal fibronectins remains to be determined. The most intriguing questions uncovered by this study concern the mechanism of control of the expression of glycosylation on the same polypeptide chain as translated in two different cell types.

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