Cell-type-specific Fibronectin Subunits Generated by Alternative Splicing*

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Multiple fibronectin mRNAs arise by alternative splicing of the primary transcript of a single gene. We describe analyses of the contribution of this alternative splicing to fibronectin subunit heterogeneity in three different cell types using antisera directed against specific segments of fibronectin. β-galactosidase-fibronectin fusion proteins produced with the λgt11 bacterial expression vector were used as immunogens. One region of alternative splicing accounts for differences in subunit size, while a second contributes to differences between the fibronectins present in blood plasma and in fibroblastic cells. We also show, however, that these two regions of alternative splicing do not account for all detectable subunits. We have also used these segment-specific antisera to show that blood platelets contain a spectrum of fibronectin subunits distinct from that found in blood plasma.

Fibronectins are large adhesive glycoproteins involved in a wide variety of biologically important phenomena including establishment and maintenance of normal morphology, cell migration during development, thrombosis and hemostasis, and oncogenic transformation (1–3). The two forms of fibronectin studied most frequently are the plasma form made by hepatocytes and the cellular form made by fibroblasts (pFN1 and cFN, respectively). These forms of FN are, in many regards, very similar in their biochemical properties. Structurally, both are disulfide-bonded dimers composed of subunits of molecular mass 250,000–280,000 that are joined by a pair of disulfide bonds near the carboxyl terminus of the molecule. Functionally, both forms have specific affinities for a variety of ligands including fibrin, gelatin, heparin, and bacteria, and both promote the attachment of cells to substrata. When functional domains of pFN and cFN (generated by limited proteolysis) are compared, the domains are found to be very similar with respect to both their affinity for the ligand and their physical properties. However, minor differences in the sizes of some domains have been noted (4, 5).

When the subunit composition of the different forms of FN are analyzed in detail, a number of differences are found. First, the two forms differ in number and types of subunits. By two-dimensional isoelectric focusing SDS-PAGE analysis, we showed previously that pFN has at least four subunits, while cFN has at least seven, of which four appear to be identical with pFN subunits, while the other three appear to be unique to cFN (6). Second, there are differences among the subunits of each form which arise from both differential post-transcriptional processing of the primary transcript and differential post-translational processing of subunits. The known post-translational modifications of FNs include N-linked complex oligosaccharides (7–15), some O-linked oligosaccharides2 (15), phosphorylation of threonine and serine (6, 17–22), and carbohydrate-linked, as well as tyrosine-linked sulfation (6, 23–27).

The gene for FN gives rise to a primary transcript that undergoes alternative splicing at, at least, two positions (28). One position of variation (V) lies between the penultimate and the last repeats of Type III sequence homology; a single exon is subdivided to yield three alternative patterns of splicing in which either 0, 285, or 360 base pairs are included in the mature transcript (29, 30). We showed previously that inclusion of the 285-base pair insert contributes to the difference in size between the larger and smaller subunits of both pFN and cFN (31). At a second position of variation, a single exon encoding a complete Type III repeat (EIII) is either included in or excluded from the mature mRNA (32–35).

This report is concerned with an analysis of the contributions of the 360 nucleotide V segment and the EIII exon to FN subunit heterogeneity. Antisera directed against these specific segments subcloned into the λgt11 bacterial expression system (36) were developed in order to determine their distribution among the various FN subunits. We also describe a novel technique for analyzing the protein variants due to RNA splicing patterns using a third antisera raised against a FN-β-galactosidase fusion protein. By immunoblot analysis, we find that inclusion of EIII is restricted entirely to cFN, and further to the three most acidic variants of this form. We also find that the 120-amino acid insert from the V region, like the 95-amino acid insert from the same region, contributes to the difference in size between subunits of both forms, but accounts neither for the differences in isoelectric point between subunits nor for any differences between FN forms. As an example of the application of these methods, we have also used the immunological reagents described in this paper to determine the nature of the FN in blood platelets.

1 The abbreviations used are: FN, fibronectin; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; cFN, cellular fibronectin; pFN, plasma fibronectin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

2 K. Skorstengaard and T. Petersen, personal communication.
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EXPERIMENTAL PROCEDURES

Cells—Primary cultures of rat hepatocytes were established by the method of Leffert et al. (37) as modified previously (6, 38). The Rat-1 fibroblast cell line (39, 40) was cultured in Dulbecco's modified Eagle's medium containing 5% calf serum (Flow Laboratories or Gibco). The Nil-8 hamster fibroblast cell line (41, 42) was cultured in Dulbecco's modified Eagle's medium containing 5% fetal calf serum (Biolabs). Both these cell lines are clonal. Human foreskin fibroblasts were provided generously by D. Wanger (Rochester University, Rochester, NY). They were cultured in Dulbecco's modified Eagle's medium containing 5% fetal calf serum at passages 14 through 16.

Purification of FN—Rat, hamster, and human plasma fibronectins were purified from fresh citrated rat and hamster plasma (Rockland Co., Gilbertsville, PA) or from fresh, heparinized, platelet-poor plasma using the same procedure as that used for the purification of y-aminofibronectin, except that elution was always carried out with 5 M urea in pH 11 CAPS buffer (10 mM cyclohexylaminopropane sulfonic acid, 150 mM NaCl, pH 11). Cellular fibronectins were purified from the culture supernatant of human foreskin fibroblasts using the same procedure as that used for the purification of y-aminofibronectin, except that elution was always carried out with 5 M urea in pH 11 CAPS buffer, aliquoted, and stored at -80°C.

Preparation and Activation of Platelets—Platelets were isolated from adult rats in a modification of the procedure of Greenberg-Sepeisky and Simons (44) with the following modifications. Blood was removed by cardiac puncture and acid/citrate/dextrose (3.8% citrate, 10 mg dextrose, 15 mg NaH2PO4 per ml) was added to 50 ml of blood cells were centrifuged at 400 x g for 5 min at room temperature in a Beckman J-13 rotor. The platelet-rich plasma was removed and recentrifuged at 1200 x g for 20 min at room temperature to pellet the platelets. After removal of the supernatant, the platelet pellet was resuspended in Hapes buffer (0.15 M NaCl, 0.0038 M Hapes, 0.0056 M glucose, 0.0038 M NaH2PO4, 0.003 M KC1, 0.001 M MgCl2, 0.35% bovine serum albumin, pH 7.35), an aliquot was removed, and the platelets counted. The platelets were resuspended at 1200 x g twice in complete Hapes buffer, and then once in incomplete Hapes buffer (minus KC1, MgCl2, and bovine serum albumin) at room temperature. After washing in incomplete Hapes buffer, the platelets were resuspended in incomplete Hapes buffer to give 1 x 10^9 platelets/ml.

Platelets were activated with thrombin (2 units/1 x 10^9 cells) at room temperature for 90 s. The reaction was stopped with a 3-fold molar excess of hirudin on ice. Thrombin and hirudin were supplied generously by S. Greenberg and R. Rosenberg (Massachusetts Institute of Technology). Platelets were then centrifuged for 5 min in an Eppendorf microfuge to separate the activated platelets from the culture supernatant. Both activated platelet and releasate fractions were stored at -35°C.

Construction of Agt11-III Recombinant Phages—Recombinant phage were constructed as described (31). In each case a segment of the subclone was digested with the restriction endonucleases EcoRI and HinfI to yield a 101-base pair fragment containing the lac z gene termination codons; this fusion protein was unstable, to maintain the -galactosidase reading frame at the 3' end of the insert since in earlier constructions with the same fragment but with EcoRI decamer linkers at the 3' end, translation did not terminate at the lac z gene termination codons; this fusion protein was unstable, and yields were poor. That the insert was in the correct reading frame and orientation was determined by sequencing (46) across the 5' boundary of the insert in Agt11-III DNA. The amino acid sequences across the N- and C-terminal boundaries are EFRDST . . . and IGVEF, respectively, whereas the EPR and EF codons are over-represented. The fusion protein therefore contains 72 of the 90 amino acids of the IIII segment. The recombinant phage-containing sequences from the heparin-binding region was clone Xrlfl (29) which contains nucleotides 516-1020 of the published rat liver cDNA. Except where otherwise noted, enzymes and linkers were purchased from New England Biolabs, Beverly, MA.

Purification of Anti-beta-galactosidase Monoclonal Antibody—The purification of beta-galactosidase-FN fusion proteins was achieved by immunoaffinity chromatography on a monoclonal anti-beta-galactosidase. The J1 hybridoma cell line was provided kindly by Tom Mason (University of Massachusetts, Amherst). Antibody was purified from ascitic fluid of gamma-irradiated BALB/c mice or F1 BALB/c x SJL/J female mice (Charles River Breeding Laboratories) or from culture supernatant provided kindly by Herman Eisen and David Rein (Massachusetts Institute of Technology). Purification of the J1 monoclonal antibody was achieved by precipitation with 50% ammonium sulfate followed by dialysis of the resuspended precipitate against 50 mM K2PO4, pH 8.0. Anti-beta-galactosidase IgG was then purified from the dialyze by DAE ion-exchange chromatography in 50 mM K2PO4, pH 5.0; the immunoglobulin was contained in the first flow-through. The column profile was monitored by SDS-PAGE analysis, and immunoglobulin-containing peak fractions were then dialyzed against 0.1 M Hpes, pH 8.0. Coupling of the J1 anti-beta-galactosidase IgG to Affi-Gel-10 (Bio-Rad) at protein/resin ratios of 5-8 mg protein/ml resin was subsequently carried out according to the manufacturer's specifications (Bio-Rad Bulletin 1085).

Purification of Recombinant Fusion Proteins—FN-beta-galactosidase fusion proteins were purified as described previously (27, 47) with the following modifications. Partial purification of the fusion proteins was achieved by ammonium sulfate precipitation and elution with 0.2 M NTE (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM EDTA) at 2 liters with four changes. The sample was then spun at 10,000 x g for 10 min, and the resulting supernatant was applied to an immunoaffinity resin made from an anti-beta-galactosidase monoclonal antibody (J1). Fusion proteins were then adsorbed to 2-3 ml of J1-Affi-Gel-10 by batch adsorption at room temperature for 2 h. The suspension of resin and protein was then poured into a 10-m1 column, and the resin was washed as follows: 20 ml of 0.1 M NTE, 20 ml of 0.2 M NTE (10 mM Tris-HCl, pH 7.5, 200 mM NaCl, 10 mM EDTA), 20 ml of 0.5 M NTE (10 mM Tris-HCl, pH 7.5, 500 mM NaCl, 10 mM EDTA), 20 ml of 0.2 M NTE. Elution of specifically bound fusion protein was achieved with 0.23 M glycine-HCl, pH 2.6. Fractions were neutralized immediately with 1 M Tris-HCl, pH 8.5. Fractions were analyzed by gel electrophoresis, and peak fractions were pooled and dialyzed against phosphate-buffered saline (250 mM NaCl, 10 mM NaHPO4, pH 7.4) and dilution was determined spectrophotometrically assuming an extinction coefficient for beta-galactosidase of OD280 = 1.0 for a solution of 1 mg/ml, verified by the Bio-Rad protein assay. Fusion proteins were stored at -80°C.

Preparation of Antisera to beta-galactosidase-FN Fusion Proteins—Soluble protein (1-2 mg/injection) was mixed with complete Freund's adjuvant (50%/v/v) and injected subcutaneously into New Zealand White rabbits (Dutchland). Two weeks later, a second injection (1-2 mg/injection) was administered with incomplete Freund's adjuvant (50%/v/v) and the rabbits were bled at weekly intervals for 4 weeks, starting 2 weeks after an immunization. The rabbits were reinjected 6 to 7 weeks after the prior immunization. Sera were screened by immunoblotting; sera positive by immunoblot analysis were obtained after five rounds of injection for the anti-III segment, while they were
obtained after only three rounds of injection for the anti-Hep rabbits.

Some antisera were raised against SDS-PAGE gel-purified fusion proteins. These antisera were reactive against polyacrylamide determinants. Such reactivity was removed by adsorption with crushed polyacrylamide gel as described previously (24).

Recombinant C1FN and pFN were prepared and characterized as described previously (41), using as immunogens FNs purified by gelatin-Sepharose affinity chromatography followed by SDS-PAGE.

Preparation of Antibodies to Peptide-carrier Conjugates—Several antisera were made to raise polyclonal antibodies against synthetic peptide-carrier conjugates using synthetic peptides containing sequences from the V region of alternative splicing coupled to a variety of proteins.

Antibodies and sera were screened by immunoblotting against both rat C1FN and pFN (native and denatured) and by immunofluorescence against Rat-1 fibroblasts. All were negative by these criteria even though strong reactions were obtained against the immunizing peptide, carrier proteins, and the chemical cross-linking reagents.

Gel Electrophoresis and Immunoblot Analysis of FN—One-dimensional analysis of samples was carried out in SDS, 5% polyacrylamide as described (48). Two dimensional analysis was carried out as described (6, 38, 49).

Polyptides were transferred from SDS-polyacrylamide gels to nitrocellulose (Schleicher & Schuell, 0.2 μm pore size, BA85) for detection by immunoblot analysis (50, 51). After electrophoresis, gels were equilibrated in 50 mM Tris-HCl, pH 6.8 for 1 h after gel-purified fusion proteins were denatured in 70% HCOOH at approximately 1 mg/ml. 100 pg to 1 μg of soluble FNs with CNBr (54), FN in CAPS buffer, pH 11, was digested with freshly prepared cyanogen bromide (CNBr) (DuPont-New England Nuclear) at 100-fold molar excess of CNBr over mole of methionine. Digestion was carried out with nitrogen, capped, and wrapped in aluminum foil. Digestion was stopped by the addition of volumes of ice-cold ddH2O, and then resuspended in a minimal volume of Laemmli’s sample buffer and boiled for 5 min. Samples were then analyzed by SDS-PAGE and immunoblot analysis.

Sizes were determined by migration relative to molecular weight markers (Bethesda Research Laboratories; ovalbumin, 43,000; chymotrypsinogen, 25,700; lactoglobulin, 18,400; lysozyme, 14,300; cytochrome C, 12,500; bovine pancreatic trypsin inhibitor, 6,200; insulin, 3,000). Autoradiographs were quantitated using an LKB laser densitometer with recording integrator.

RESULTS

The Nature of FN Subunit Heterogeneity—By high resolution one-dimensional SDS-PAGE and two-dimensional isoelectic focusing SDS-PAGE analysis, we previously defined a minimum number of subunits for rat fibronectins (See Ref. 6 and Fig. 1, Panel A). pFN, purified from whole rat plasma or from the conditioned medium of primary cultures of rat hepatocytes, has at least four resolvable subunits, designated P1α, P1β, P2α, and P2β, and C1FN and pFN purified from the conditioned medium of the Rat-1 fibroblastic cell line consists of at least seven discernable molecular species designated C1α, C1b, C1c, C1d, and C2a, C2b, and C2c. pFN and C2c share at least four subunits, namely, C1c/P1α, C1d/P1b, C2b/P2α, and C2c/P2b, and rat C2FN has at least three subunits that are not present in pFN (C1a, C1b, and C2a).

The known post-translational modifications of FN appear to contribute little to the observed diversity of subunits (6). A second explanation for these variants is offered by the results of recombinant DNA analysis, which has shown that the gene for FN gives rise to a primary transcript that is alternatively spliced (28–35) as shown in Fig. 1, Panel B. In this report we have analyzed the contribution of alternative RNA splicing to the structural diversity of FN subunits.

Variations Due to the EIII Segment—Several polyclonal

A PROTEIN VARIANTS

B SPlicing VARIANTS

FIG. 1. Schematic of rat fibronectin protein variants and RNA splicing patterns (see Refs. 6 and 28). Panel A, FN protein variants. White spots are unique to pFN, black spots are unique to C1FN, and shaded spots are common to both forms. Panel B, RNA splicing variants. Black segments represent exons that are always present in the mature mRNA, and white segments represent exons that are alternatively spliced.
antiseras raised against a β-galactosidase-EIII fusion protein were used to identify FN subunits containing this segment. One of the anti-EIII antiseras recognizes only rat cFN by immunoblot analysis of one-dimensional gels (Fig. 2, Tracks 1–12). Rat pFN and hamster or human fibronectins are not recognized by this particular serum (Fig. 2, Tracks 7, 9–12). A second polyclonal antiserum raised against the same fusion protein confirms the restricted reactivity against cFN (Fig. 2, Tracks 17 and 18). In rat cFN, both the C1 and C2 subunits are recognized by these sera, indicating that the EIII sequence occurs in subunits of both sizes unlike the V region, which occurs only in the larger subunits (See Ref. 31).

By two-dimensional immunoblot analysis of rat cFN, the anti-EIII antiserum recognizes only a limited number of subunits, namely C1a, C1b, and C2a (Fig. 3, Panels A and B). cFN subunits C1c, C1d, C2b, and C2c are not recognized by the antiserum to the E111 segment and thus do not contain this sequence. This is in agreement with our earlier conclusion that the more basic subunits of cFN are the same as the four subunits of pFN (6).

The C2a spot detected with the anti-EIII antiserum and the C2b spot were not detected previously. Both are minor spots and C2a is resolved poorly from C1b. To determine whether these spots are the result of degradation of C1a or C1b, nitrocellulose filter transfers of two-dimensional gels of rat cFN were probed with the anti-V95 antiserum we described previously (31). This antiserum recognizes FN spots containing sequences from the V region which contributes to the difference in size between C1 subunits and C2 subunits of rat cFN.

FIG. 2. Immunoblot analysis of FN subunits. FNs purified by gelatin-Sepharose affinity chromatography (1–2 μg/track) were separated by SDS-PAGE and transferred to nitrocellulose filters. Transferred proteins were stained first with India Ink (Tracks 1–6, 13, 14) and then with antiserum against β-galactosidase-FN fusion proteins (Tracks 7–12, 17–18, anti-EIII; Tracks 19 and 20, anti-Hep). Specific binding of antibodies to FN subunits was detected with 125I-protein A followed by autoradiography. The anti-EIII antiserum used on Tracks 7–12 is from a different rabbit than that used on Tracks 17 and 18. These two sera were used interchangeably as both recognize the same rat FN subunits on blots. The preimmune serum used on Tracks 15 and 16 is from the same rabbit as the immune anti-EIII serum used on Tracks 17 and 18. Tracks 1 and 7, rat pFN; Tracks 2 and 8, rat cFN; Tracks 3 and 9, hamster pFN; Tracks 4 and 10, hamster cFN; Tracks 5 and 11, human pFN; Tracks 6 and 12, human cFN; Tracks 13, 15, 17, and 19, rat pFN; Tracks 14, 16, 18, and 20, rat cFN.

FIG. 3. Two-dimensional immunoblot analysis of rat cFN subunits. Rat cFN purified by gelatin-Sepharose affinity chromatography was separated on two-dimensional gels and transferred to nitrocellulose filters. Transferred proteins were stained first with India Ink (Panels A and C) and then with antiseras directed against β-galactosidase-FN fusion proteins (Panel B, anti-EIII; Panel D, anti-V95). Specific binding of antibodies to cFN subunits was detected with 125I-protein A. cFN subunits C1a, C1b, and C2a are recognized by the anti-EIII serum (cf. Panels A and B); the C1 subunits but not the C2 subunits are recognized by the anti-V95 serum (cf. Panels C and D).

FIG. 4. Schematic of chemical cleavage-immunoblot assay. The schematic shows FN around the V region of sequence variation. The black segment represents the Type III sequences N-terminal to the V region. The anti-Hep antiserum, which was raised against a β-galactosidase-FN fusion protein, binds to this region of FN. Cleavage of FN with cyanogen bromide at methionine residues (M) yields five fragments that overlap with the “Hep” sequences: fragment A (56 amino acids), fragment B (18 amino acids), and fragments C (373 amino acids), D (268 amino acids), and E (243 amino acids), representing, respectively, the inclusion of 0, 120, and 95 amino acids from the V region. Resolution of the CNBr digests of FN on 15% SDS-PAGE gels followed by immunoblot analysis is shown at the bottom of the figure. Fragments C, D, and E are clearly detected by the anti-Hep antiserum; Fragments A and B are not detected readily because they contain inadequate immunological determinants to be recognized and/or because they are too small to be retained on the nitrocellulose filters.
rat cFN. Anti-V95 reactive material is found in positions corresponding only to subunits C1a, C1b, C1c, and C1d; no anti-V95 reactive material comigrated with subunits C2a, C2b, or C2c (cf. Panel C with Panel D, Fig. 3). Therefore, the C2a subunit does not represent a degradation product of the C1a or C1b subunits, as it contains sequences from the E111 exon (Fig. 3, Panel B) but none from the V region (Fig. 3, Panel D). Similarly, the C2b spot which contains neither insert is not a degradation product of any C1 subunits.

These results demonstrate that the sequence encoded by the EIII exon is restricted entirely to cFN. Furthermore, this segment is only found in the larger and more acidic subunits of cFN.

Variations Due to the V Segment—We turned next to a consideration of the contribution to FN subunit heterogeneity by the second region of variation, the V region. We have previously described the distribution of the 95-amino acid segment of the V region using an antiserum raised against a fusion protein containing this segment (31). However, we have been unsuccessful in several attempts to raise antibodies against the amino-terminal 25-amino acid segment of this region as described under “Experimental Procedures.”

Therefore, we designed an assay which makes use of a polyclonal antiserum (anti-Hep) raised against another β-galactosidase-FN fusion protein. This fusion protein contains a stretch of Type III sequence from the carboxyl-terminal heparin binding domain of FN that lies between the EIII and the V regions (see “Experimental Procedures”). Unlike the anti-EIII antiserum, the anti-Hep antiserum is directed against sequences present in all FN subunits from both pFN and cFN (Fig. 2, Tracks 19 and 20; two-dimensional immunoblots not shown).

The principle of the assay is outlined in Fig. 4. Purified FNs (or mixtures of proteins containing FN) are first cleaved at methionine residues with cyanogen bromide. The digestion products, which include FN fragments of known size (deduced from primary structural information) are separated by one-dimensional SDS-PAGE, transferred to nitrocellulose filters, and then probed with the anti-Hep antiserum. Since this antiserum binds to FN sequences adjacent to the V region, it will recognize fragments which extend into or beyond the V region. The fragments that are detected by the anti-Hep antiserum will reflect the presence of either the VO, V95, or V120 segments and the relative abundance of each alternative.

The advantages of this assay are severalfold. First, it allows detection of all three sequence variants at the V region using a single antiserum rather than a different serum directed against each alternative. Second, the assay can be used on heterogeneous mixtures of proteins (whole cell lysates, extracellular matrix, plasma, tissue samples) in which FN is only a minor component, so long as the protein is present in sufficient amounts (1–10 μg/analysis).

Cleavage of FN with CNBr to completion should yield five fragments that overlap with the “Hep” sequence: fragment A (56 amino acids), fragment B (18 amino acids), and fragments C (373 amino acids), D (268 amino acids), and E (243 amino acids) representing, respectively, the inclusion of 0, 120, and 95 amino acids from the V region (Fig. 4). Immunoblot analysis of rat FNs cleaved with CNBr and probed with the anti-Hep antiserum is shown in Fig. 5. We did not detect fragments A and B presumably because they contain inadequate immunological determinants to be recognized and/or are too small to be retained on the nitrocellulose filters. However, the three fragments of interest (C, D, and E) were readily detected as well as higher molecular weight material that presumably represents incomplete digestion products (Fig. 5, Panel A). Both pFN and cFN yield fragments C, D, and E, indicating that both forms of FN contain all three of the V region variants. However, the relative ratios of C/D/E differ between forms. The ratios of the different bands were determined by densitometry. Whereas the ratio of C/D/E (0:120:95) is approximately 1.5:1:1.5 in cFN (Panel A, Track 1), it is approximately 1.5:1:1.7 for pFN (Panel A, Track 2). In cFN, subunits containing V region sequences (either V95 or V120) predom-
Fig. 6. Immunoblot analyses of platelet FN. Panel A, platelet extracts and FNs purified by gelatin-Sepharose affinity chromatography were resolved by SDS-PAGE on 5% gels and transferred to nitrocellulose filters. Transferred proteins were stained first with India ink (Tracks 1–6) and then with antiserum against β-galactosidase–FN fusion proteins (Tracks 7–12, anti-Hep; Tracks 13–18, anti-EIII; Tracks 19–24, anti-95) and 122I-protein A. Tracks 1, 7, 13, and 19, rat cFN; Tracks 2, 8, 14, and 20, rat pFN; Tracks 3, 9, 15, and 21, whole platelet lysate; Tracks 4, 10, 16, and 22, activated platelet residue; Tracks 5, 11, 17, and 23 activated platelet releasate; Tracks 6, 12, 18, and 24, rat cFN. Panel B, purified rat cFN and whole platelet extracts were resolved by SDS-PAGE on 5% gels. FN bands were excised from the gels and digested with cyanogen bromide. The resulting digestion products were separated on 15% SDS-PAGE gels and detected with the anti-Hep antiserum and 122I-protein A. Fragments C, D, and E represent no V region insert, 120- (25 plus 95) amino acid V region insert, and 95-amino acid V region insert, respectively. Tracks 1 and 4, total pFN digest; Tracks 2 and 3, platelet FN digest.

Subunit Composition of Platelet FN—While platelets are known to contain FN in their α-granules, the origin and nature of this FN is not known. We have used the anti-Hep, anti-EIII, and anti-V95 antisera, along with the chemical cleavage-immunoblot assay described above to characterize the FN found associated with platelets in both their resting and activated states. Fig. 6 shows the results of immunoblot analysis of whole platelet extracts, thrombin-activated platelet residues, and the material secreted from platelets upon thrombin activation. Panel A, Tracks 7–12 shows the results of immunoblot analysis of these various platelet samples with the anti-Hep antisera; in all three platelet fractions FN is detected by the anti-Hep antiserum (Tracks 9–11). This anti-Hep reactive material migrates in this gel system with a molecular mass which appears very slightly greater than that of rat pFN or cFN (cf. Tracks 8 and 12 with Tracks 9–11). When duplicate filters are probed with the cFN-specific anti-EIII antisera (Tracks 13–18), material migrating at a position slightly above or with that of rat cFN is detected. Thus,

Furthermore, within each of the spots P1a and P1b there are two species, namely, those representing the inclusion of either the 95- or the 120-amino acid inserts. We also conclude that the doubling of spots seen for rat pFN subunits P1a and P1b, P2a and P2b, and by deduction, cFN subunits C1a and C1b, C1c and C1d, and C2b and C2c, is not due to the inclusion of the 25-amino acid segment from the V120 region. This doubling must have its origins elsewhere.

To determine the contribution of the 25-amino acid portion of the V120 region to FN subunit heterogeneity, we have used the chemical cleavage-immunoblot assay in the following manner. pFN subunits were separated by two-dimensional gel electrophoresis, and the individual subunits were visualized by staining with Coomassie Brilliant Blue. Subunits Pla, Plb, Pza, and P2b were cut from the gels, digested with cyanogen bromide, and the digestion products of each subunit were then separated by SDS-PAGE on a 15% gel. Following transfer to nitrocellulose, the filters were probed with the anti-Hep antiserum; in all three platelet fractions FN is detected by the anti-Hep antiserum (Tracks 9–11). This anti-Hep reactive material migrates in this gel system with a molecular mass which appears very slightly greater than that of rat pFN or cFN (cf. Tracks 8 and 12 with Tracks 9–11). When duplicate filters are probed with the cFN-specific anti-EIII antisera (Tracks 13–18), material migrating at a position slightly above or with that of rat cFN is detected. Thus,
the FN that is associated with platelets is not pFN. Rather, it is closely related to the FN synthesized and secreted by fibroblasts (cFN) because it contains sequences from the EIII region of FN.

Immunoblot analysis of rat cFN, pFN, and platelet extracts with the anti-95 antiserum shows that the FN associated with platelets contains sequences from the V region (Fig. 6, Tracks 19–24). Only the larger subunits of platelet FN are detected with this antiserum, which is consistent with the results seen when rat pFN and cFN are analyzed with this same antiserum.

When FN from platelet extracts is analyzed by the chemical cleavage-immunoblot assay for the presence and relative abundance of sequences from the V region, CNBr fragments C, D, and E are detected with the anti-Hep antiserum (Fig. 6, Panel B).

In summary, immunoblot analysis of platelet-derived FN shows that it contains all three of the V region variants as well as the Type III repeat encoded by the EIII exon. Platelet-derived FN is not the same as the FN found in blood plasma by virtue of the presence of the EIII sequence; it is more like the cFN produced by fibroblasts.

**DISCUSSION**

The data presented here provide information on the contribution of alternative RNA splicing to fibronectin subunit heterogeneity in three different cell types. As shown in Fig. 1, two-dimensional gel electrophoresis resolves at least seven distinguishable FN subunits, while the two reported positions of alternative splicing can produce up to six variant mRNAs. The question under examination is whether these known splicing variants account for the observed heterogeneity. The answer is that they do so only in part. While the splicing variants clearly account for size differences between FN subunits and for differences between the FNs produced by cFN and pFN, they do not account for all the observed heterogeneity. Furthermore, as shown here, the heterogeneity is even greater than previously suspected. Therefore, there must be other elements contributing to the heterogeneity.

Using antisera specific for the variant regions (anti-V95 and anti-EIII), we have shown here and elsewhere (31) that inclusion of the V95 difference segment accounts for the two size classes of plasma FN subunits and for size differences among cFN subunits, and we show here that the EIII segment is confined to those subunits unique to cFN. That is, inclusion of the exon encoding the EIII segment is responsible for a difference between cFN and pFN. This result is consistent with S1 nuclease and RNase protection analyses showing that this exon is included in mRNA by fibroblasts and other cell lines but not by liver or hepatocytes (32, 33). We had hoped to complete this analysis using antisera specific for the V25 segment but, despite extensive efforts to produce such sera using a variety of immunization and screening protocols (see "Experimental Procedures"), we were unable to obtain reactivity against this segment of FN. It appears that antisera to synthetic peptides that cross-react with the native or denatured parent molecule are not always as readily obtained as is sometimes supposed.

We, therefore, developed an alternative method for approaching this problem, using antisera specific for segments of FN adjacent to regions of variation (Figs. 4–6). Using this method we were able to detect and quantitate the presence or absence of the V25 segment. We found that both fibroblasts and hepatocytes contain FN subunits with and without this segment. Furthermore, we showed that V25" and V25" subunits were present together in single spots on two-dimensional gels. Therefore, the V25 segment cannot provide an explanation for that observed heterogeneity which is not explained by the V95 or EIII segments.

At this point, our interpretation of the observed pattern of spots on two-dimensional gels of carbohydrate-free FNs (Fig. 1, Panel A) is as follows. P2a and P2b contain none of the inserts, as shown by their failure to react with anti-V95 or anti-EIII and by the absence from these spots of a V25* variant (Fig. 5). Addition of V95 but not of EIII gives the larger molecular mass pFN spots, P1a and P1b. Both of these spots contain both V25* and V25" variants (Fig. 5). Addition of EIII but not V95 gives rise to the cFN-specific spot, C2a, while addition of both V95 and EIII gives rise to the cFN-specific subunits C1a and C1b (Fig. 3). If one assumes that C1a and C1b each contain both V25* and V25" variants like P1a and P1b then the three cFN-specific spots, C1a, C1b, and C2a appear to correspond with the three pFN spots P1a, P1b, and P2b, but differ from them by the inclusion of the EIII segment which is absent from pFN. cFN and pFN also differ in the relative abundance of V region variants (in cFN, V25 variants predominate, while in pFN, V95 and V" variants are present in roughly equivalent amounts). We do not detect a cFN spot (V-, EIII*) analogous to P1a (V-, EIII*); it would lie to the acidic side of C2a and may simply be present at undetectable levels (note that C1a is much less prevalent than C1b).

While these interpretations are satisfactory, they do not account for the pairs of spots P1a/P1b, P2a/P2b, and C1a/C1b. The members of each of these pairs are indistinguishable in their content of V25, V95, and EIII. How can one account for this unexplained heterogeneity? As we showed previously, neither covalently linked phosphate, nor sulfate, nor N-linked complex oligosaccharides can explain the differences between these subunits (6). Preliminary evidence suggests that neither heterogeneity in terminal sialic acid residues nor the presence of O-linked sugars can account for this "doubling" of spots either, as removal of sialic acid from FNs synthesized in the presence of tunicamycin does not reduce the complexity of pFN subunits further. It is possible that some other previously undetected post-translational modification contributes to structural differences among FN subunits. While there is currently no evidence to support the notion that such a modification exists, it nonetheless remains an open possibility.

A second, and more attractive hypothesis, is that yet other regions of alternative mRNA splicing exist and provide an explanation for the unexplained heterogeneity in FN subunits. Such a region of alternative splicing would need to occur in both fibroblasts and hepatocytes to account for the extra spots observed in both cFN and pFN. If such a variation were to exist, it would increase the number of potential subunits of rat FN to 12 which, assuming the presence of two different subunits (V25* and V25") in each of the spots C1a, C1b, P1a, and P1b and the existence of a minor acidic cFN-specific spot (V-, EIII*), would fit with the known heterogeneity. We are currently searching for a variation with the requisite properties.

We have also applied our antisera in an analysis of platelet FN. Platelets are known to contain approximately 4000 molecules of FN per platelet, enclosed in a granules (55–57). These are secretory granules released when platelets are activated. During this same activation process, platelets acquire approximately 10⁶ FN receptors per platelet (58). It has been a puzzle as to why platelets should contain a small number of...
FN molecules, sufficient to bind only a tiny fraction of their own receptors, especially when one considers that activated platelets are exposed to μM concentrations of pFN, sufficient to saturate the platelet receptors (16). We show here that platelet FN is distinct from pFN. In addition to the V95 and V25 segments, it contains the E111 segment (Fig. 6). In this respect it resembles fibroblast cFN rather than pFN. Platelet FN appears slightly larger than fibroblast cFN on gels (Fig. 6), but this could be due to glycosylation differences. It is not straightforward to eliminate the carbohydrates from platelet FN since this FN represents a stored pool; platelets lack a nucleus and make virtually no protein. Therefore one cannot use tunicamycin to block glycosylation. It is, therefore, satisfying to be able to analyze the pattern of RNA splicing which gave rise to platelet FN, using segment-specific antisera to probe the end product. We presume this platelet FN was synthesized in the megakaryocyte precursors of platelets since it clearly is not derived from pFN. This result raises the intriguing possibility that platelet FN (and cFN) which include the E111 segment may possess functions lacking from pFN. Possibilities include the ability to nucleate fibril formation and/or bind to specific ligands such as other extracellular molecules or cell surface receptors. The antisera we have described here and elsewhere should prove useful in further analyses of the functions of the regions of variation among FN subunits and types.

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