Probing Molecular Polymorphism of Fibronectins with Antibodies Directed to the Alternatively Spliced Peptide Segments†

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ABSTRACT: Molecular heterogeneity of fibronectins (FNs) isolated from plasma, cultured fibroblasts, and placenta was studied with site-specific antibodies recognizing alternatively spliced peptide segments, termed ED-A and IIICS/Δ_2. The antibodies were raised in rabbits by immunization with synthetic peptides. Neither the ED-A nor the IIICS/Δ_2 extra peptide segment was present in the major subunits of plasma FN, although a minor subunit contained the latter extra segment. Cellular FN consisted of at least four subunits differing in size of the fragments generated by cleavage of the C-terminal region with cathepsin D. These fragments were distinct from each other in the reactivity with anti-ED-A and anti-IIICS/Δ_2 antibodies, suggesting that all combinations of the presence or absence of the extra segments were produced by cultured fibroblasts. Placental FN was more heterogeneous than plasma and cellular FNs, consisting of five, or probably more, subunits. Among these, the two smaller subunits appeared to be closely similar to the major subunits of plasma FN, whereas the other subunits were more related to those of cellular FN in the size of cathepsin D cleaved C-terminal fragments and in the reactivity with anti-peptide antibodies. These results, taken together, indicate that the FNs produced by different tissues or cell types are distinct from each other in the number and types of subunits, which are partly, if not all, defined by alternative splicing at the ED-A and IIICS regions.

Fibronectins (FNs)[1] are multifunctional adhesive glycoproteins present in the extracellular matrix and various body fluids. FNs consist of two identical or nonidentical subunits with M_r of about 250 000, which are folded into a series of protease-resistant domains with characteristic binding capabilities [for recent reviews, see Yamada (1983), Mosher (1984), Hakomori et al. (1984), Hynes (1985), and Ruoslahti (1988)]. Sequence analyses at the protein and cDNA levels have revealed that FN polypeptides are made up of three types of internally homologous repeats referred to as type I, type II, and type III, each encoded by a single, or at most a pair of, exon(s) (Petersen, 1985; Hynes 1985; Patel et al., 1987; see also Figure 1).

FNs isolated from different tissues or cell types have been shown to be similar in many molecular and biological properties but differ slightly in the subunit size (Yamada & Kennedy, 1979; Hayashi & Yamada, 1981; Paul & Hynes, 1984; Sekiguchi et al., 1985). FNs synthesized by cultured fibroblasts and other cell types, collectively referred to as "cellular FN", consist of multiple subunits that are significantly larger than the two major subunits of "plasma FN". Nucleotide sequence analysis of the cDNAs encoding cellular and plasma FNs and of the FN gene have revealed that multiple FN isoforms arise from a single gene by alternative pre-mRNA splicing at three distinct regions, named ED-A, ED-B, and IIICS (Schwarzbauer et al., 1983, 1985, 1987; Kornblihtt et al., 1984a,b, 1985; Sekiguchi et al., 1986a; Zardi et al., 1987; Gutman & Kornblihtt, 1987; see also Figure 1). The ED-A and ED-B regions are considered to be included in cellular FN but excluded from plasma FN (Kornblihtt et al., 1984a; Paul et al., 1986; Peters et al., 1986; Borsi et al., 1987; Norton & Hynes, 1987; Vartio et al., 1987).

The alternative splicing at the IIICS region generates up to five different mRNA variants due to the internal donor and acceptor sites for optional splicing within a coding exon (Schwarzbauer et al., 1983; Bernard et al., 1985; Umezawa et al., 1985; Sekiguchi et al., 1986a; see Figure 1). Comparison of the amino acid sequences of human FNs at the IIICS region determined by direct protein sequencing (Garcia-Pardo et al., 1985, 1987; Pande et al., 1987) and those deduced from the nucleotide sequences of FN cDNAs (Kornblihtt et al., 1985; Umezawa et al., 1985; Sekiguchi et al., 1986a) indicated that the C-terminal 31 residues of the 120 amino acid IIICS sequence (referred to as IIICS/Δ_2 in this paper) could be included in cellular FN but are excluded from plasma FN.

Despite the importance of differential pre-mRNA splicing in molecular diversity of FNs, it has not yet been clear what kinds of isoforms, with or without each alternatively spliced peptide segment, are expressed in different tissues and cell types. In the present study, we analyzed molecular heterogeneity of three different forms of human FNs isolated from plasma, placental tissue, and cultured fibroblasts with site-specific antibodies directed to the ED-A and IIICS/Δ_2 peptides. The results obtained clearly show that these FNs differ

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[1] Abbreviations: FN, fibronectin; ED, extra domain; IIICS, type III connecting segment; BSA, bovine serum albumin; CAPS, 3-(cyclohexylamino)propanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; ECH-Sepharose, epoxy-activated Sepharose derivatized with 6-aminohexanoic acid; PAGE, polyacrylamide gel electrophoresis; PBS, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, and 2.7 mM KCl, pH 7.4.

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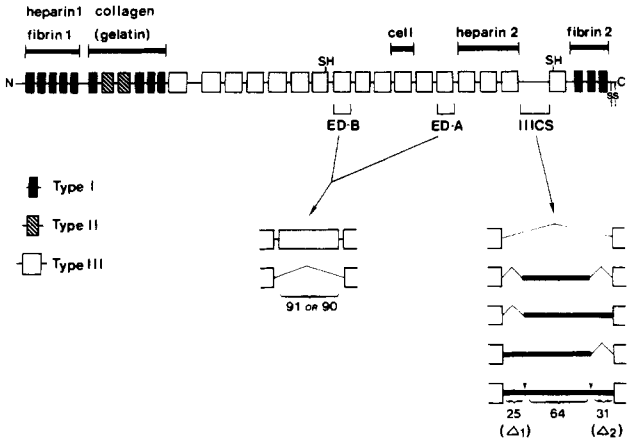

FIGURE 1: Model for the modular structure of FNs and its variations due to alternative pre-mRNA splicing. Alternative splicing at the ED-A and ED-B exons results in the inclusion or skipping of these exons, each encoding an entire type III homology sequence. Alternative splicing at the IIICS region generates five variants due to the subdivision of the particular exon. Location of the domains binding to cell, heparin, and fibrin is indicated by brackets above the modular structure model.

significantly in the occurrence of the extra peptide segments as well as the combination thereof.

Experimental Procedures

Peptide Synthesis. Peptides were synthesized with an automated peptide synthesizer (Model 430A, Applied Biosystems, Foster City, CA). Deprotection and release of the peptides from the solid-phase support matrix were accomplished with anhydrous hydrogen fluoride as suggested by the supplier. After extraction with 2 M acetic acid, the peptides were chromatographed on a column of Bio-Gel P-4 in 1 M acetic acid. The synthesized peptides were verified by the amino acid analysis.

Production of Anti-Peptide Antibodies. The synthetic peptides were conjugated to keyhole limpet hemocyanin (Calbiochem, La Jolla, CA) by the glutaraldehyde method of Kagan and Glick (1979). Briefly, each peptide (6 mg) was dissolved in 1 mL of 0.1 M sodium phosphate (pH 7.5) and mixed with an equal volume of the same buffer containing hemocyanin (approximately 10 mg/mL). One milliliter of 20 mM glutaraldehyde was then added dropwise under gentle agitation, and the reaction was allowed to proceed for 30 min at room temperature. The peptide–hemocyanin conjugate was desalted on a Sephadex G-50 column equilibrated with 0.1 M sodium phosphate. Protein-containing fractions excluded from the column were pooled and dialyzed against PBS. For immunization, the peptide–hemocyanin conjugates were emulsified in an equal volume of Freund's complete adjuvant and injected intradermally at multiple sites on the back of white rabbits. After two more injections at 2-week intervals, the rabbits were bled for antiserum.

Purification of Anti-Peptide Antibodies. The antibodies were purified on affinity columns of the antigenic peptides immobilized on ECH-Sepharose (Pharmacia, Uppsala, Sweden). Each peptide (approximately 7 mg), dissolved in 7 mL of 1 mM HCl (pH 4–5), was immobilized on ECH-Sepharose (approximately 2 mL of wet gel) with the aid of water-soluble carbodiimide as suggested by the supplier. The peptide columns were washed once with 0.2 M glycine hydrochloride (pH 2.8) and then with PBS. For affinity purification of the anti-peptide antibodies, each antiserum (25–30 mL) was applied to the appropriate peptide column (bed volume 1.7–1.8 mL, equilibrated with PBS) and washed extensively with PBS, and the bound antibodies were eluted with 0.2 M glycine hydrochloride (pH 2.8). The eluate was immediately neutralized with 1 M Tris-HCl (pH 9.0) to bring the pH to around 7. The antibodies thus purified were dialyzed against PBS at 4 °C and stored at −85 °C.

Purification of FNs. Plasma FN was purified on a gelatin–Sepharose column from the supernatant of concentrated factor VIII fraction of human serum by precipitation with glycine as described previously (Sekiguchi et al., 1983). Cellular FN was purified from the conditioned medium of human lung fibroblast WI-38, which was supplied by Japanese Cancer Research Resources Bank (Tokyo, Japan), as described previously (Sekiguchi et al., 1985). Placental FN was purified from a fresh term placenta according to Zhu et al. (1984) and Isemura et al. (1984). Briefly, the fresh placenta was immediately perfused with ice-cold PBS through the cord artery to remove blood. The placenta was then dissected to select healthy villi from decidua and any necrotic tissues. The tissue was further washed in ice-cold PBS and then blended for 2 min in PBS containing 1 mM PMSF and 1 mM EDTA. After centrifugation at 15000*g* for 15 min at 4 °C, the pellet was washed three more times by blending in PBS containing 1 mM PMSF and 1 mM EDTA and subsequent centrifugation to remove the residual blood. The pellet was then blended in 4 volumes of PBS containing 2 M urea, 1 mM PMSF, and 1 mM EDTA, and the resulting thin slurry was shaken overnight at 4 °C to extract FN. After centrifugation at 20000*g* at 4 °C, the supernatant was diluted with 15 volumes of ice-cold PBS containing PMSF and EDTA and then applied to a gelatin–Sepharose column (bed volume 30 mL) equipped with a precolumn filled with underivatized Sepharose CL-4B. The gelatin–Sepharose column was sequentially washed with (1) PBS containing 1 mM PMSF and 1 mM EDTA, (2) PBS containing 1 M NaCl, 1 mM PMSF, and 1 mM EDTA, and (3) PBS containing 0.8 M urea, 1 mM PMSF, and 1 mM EDTA. The bound FN was eluted with PBS containing 4 M urea, 1 mM PMSF, and 1 mM EDTA, extensively dialyzed against 2.5 mM CAPS buffer containing 0.5 mM EDTA (pH 10.5), and then stored at −85 °C. The protein concentration of FNs was determined from the UV absorbance at 280 nm, assuming that the absorption coefficient of 0.1% FN solution at pH 10.5 is 1.31 cm^{-1}.

Cathepsin D Digestion of FNs. Purified FNs were digested with cathepsin D essentially as described by Richter et al. (1981) at an enzyme-to-substrate ratio of 1:200. The digestion was performed at 37 °C for 3 h and terminated by addition of a 10 times molar excess pepstatin A and an appropriate volume of 1 M Tris-HCl (pH 9.0) to bring pH back to about 7. The digests were lyophilized and analyzed by SDS–PAGE and subsequent immunoblot analysis.

SDS–PAGE and Immunoblot Analysis. SDS–PAGE was performed on 6% (for intact FNs) or 8% (for cathepsin D digests) gels by the method of Laemmli (1970). Samples were reduced with 5% 2-mercaptoethanol in the presence of 2% SDS. After electrophoresis was completed, the proteins in the gel were electrophoretically transferred to nitrocellulose membrane (Bio-Rad, Richmond, CA) according to Towbin et al. (1979). The nitrocellulose membrane was first stained with Fast Green to visualize proteins (Woodcock-Mitchell et al., 1982), blocked with PBS containing 2% BSA for 2 h, and then incubated with antibodies at an appropriate dilution in PBS containing 2% BSA for 2 h. When the first antibody was mouse monoclonal antibody, the nitrocellulose membrane was briefly washed with PBS containing 0.2% BSA and then incubated with rabbit anti-mouse immunoglobulin antibodies

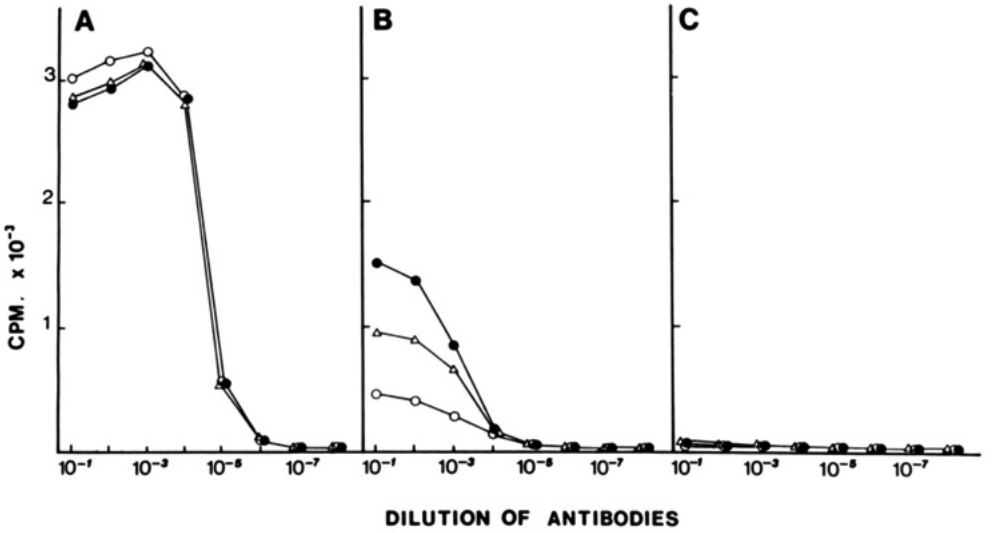

FIGURE 2: Solid-phase binding assay of the anti-peptide antibodies. Flat-bottom 96-well plates were coated with 10 μg/mL plasma (open circles), cellular (closed circles), and placental (open triangles) FNs and then incubated with serially 10-fold diluted polyclonal anti-FN antibodies (panel A), anti-IIICS/Δ_2 peptide antibodies (panel B), or anti-ED-A peptide antibodies (panel C).

(Cappel Worthington Biochemicals, Malvern, PA) at 1:2500 dilution for 1 h in PBS containing 2% BSA. After washing with PBS containing 0.2% BSA, the nitrocellulose membrane was incubated with ^{125}I-protein A (Amersham Japan, Tokyo, Japan) in PBS containing 2% BSA for 30 min, washed extensively with PBS containing 0.2% BSA, air-dried, and exposed to a Kodak XAR-5A film at −85 °C. Monoclonal anti-FN antibody FN8-12, which is directed to the C-terminal fibrin-binding ("fibrin 2") domain, was a generous gift from Masahiko Katayama (Takara Shuzo Co., Kyoto, Japan). Radioactivity associated with each protein band was estimated by a Fujix Bio-Image Analyzer BA100 (Fuji Photo Film Co., Kanagawa, Japan).

Solid-Phase Binding Assay. Solid-phase antibody binding assay was performed as described previously (Sekiguchi et al., 1982). Flexible assay plates (Falcon no. 3912, Becton Dickinson, Oxnard, CA) were coated with purified FNs (10 μg/mL in PBS) at 50 μL per well for 12–16 h. The plates were blocked with PBS containing 2% BSA for 4–6 h, incubated with antibodies at 10-fold serial dilutions in PBS containing 2% BSA for 12–14 h at 4 °C, washed with PBS containing 0.2% BSA, and then incubated with ^{125}I-protein A (2×10^5 cpm per well) in PBS containing 2% BSA for 30 min. The plates were extensively washed with PBS containing 0.2% BSA and air-dried, and the radioactivity retained in each well was determined by a well-type γ counter (Aloka ASP-120, Aloka, Tokyo, Japan).

Results

Preparation of Anti-Peptide Antibodies. Antigenic sequences within the IIICS/Δ_2 and ED-A extra regions were predicted by the algorithm of Hopp and Woods (1981), and peptides with the following sequences were chemically synthesized; YEEIQIGHIPREDVDY (the IIICS/Δ_2 peptide) and YSSPEDGIHELFPAP (the ED-A peptide). These peptides were conjugated to keyhole limpet hemocyanin and used as immunogens to raise antibodies in rabbits. The titer of the antibodies, measured as the reactivity with intact cellular FN, was monitored after each round of immunization by immunoblot analysis. A high titer of antibodies recognizing cellular FN was elicited in rabbits immunized with the IIICS/Δ_2 peptide, although a relatively low titer of antibodies was obtained from rabbits immunized with the ED-A peptide. Another peptide, TNIDRPKGLAFTDVD, representing the N-terminal 15 residues in the 90 amino acid sequence of the ED-A region, was also synthesized and tested but was unsuccessful in raising antibodies reactive with cellular FN (data not shown). The two anti-peptide antibodies capable of recognizing cellular FN were affinity purified on the immobilized peptide columns and used in the following experiments.

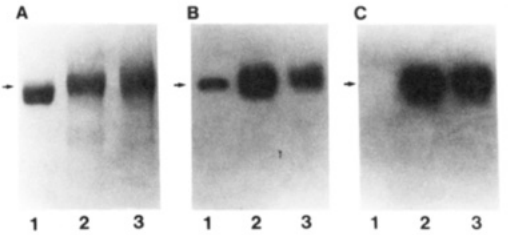

FIGURE 3: Immunoblot analysis of the different forms of FNs with anti-peptide antibodies. FNs (3 μg of protein) were separated on a 6% SDS–polyacrylamide gel, transferred to a nitrocellulose membrane, stained for protein with Fast Green (panel A), and then immunostained with anti-IIICS/Δ_2 antibodies (panel B) or anti-ED-A antibodies (panel C). Longer exposure to an X-ray film was required for immunostaining with anti-ED-A antibodies than with anti-IIICS/Δ_2 antibodies. Lane 1, plasma FN; lane 2, cellular FN; lane 3, placental FN. The arrow points to the position of the upper rim of the plasma FN band immunostained with anti-IIICS/Δ_2 antibodies.

Solid-Phase Binding Assay. Reactivities of the anti-peptide antibodies with the different forms of FNs were first tested by solid-phase binding assay (Figure 2). The anti-IIICS/Δ_2 antibodies were capable of binding to all three forms of FNs, i.e., plasma, cellular, and placental FNs, although the amounts of the bound antibodies differed significantly among these FNs (Figure 2B). Cellular FN was most reactive, binding nearly three times more antibodies than plasma FN. Placental FN also bound nearly twice as many antibodies as plasma FN. These differences should not be due to the difference in the amounts of the FNs adsorbed on the assay plates, because the polyclonal anti-FN antibodies bound equally to all of these three FNs (Figure 2A).

In contrast, the anti-ED-A antibodies were capable of binding to none of these FNs adsorbed on the plastic surface (Figure 2C). Since the antibodies could react with cellular FN blotted onto a nitrocellulose sheet (see below), these results suggest that the ED-A epitope within the FN molecule adsorbed on the plastic surface is either not exposed so that the antibodies cannot gain access to it or does not assume an appropriate conformation recognizable by the antibodies which

Table I: Quantitative Immunoblot Analysis with Anti-Peptide Antibodies

FN types	bound radioactivities[a] anti-IIICS/Δ_2 antibodies	anti-ED-A antibodies
plasma FN	2090 (28)[b]	<50 (<5)
cellular FN	7407 (100)[c]	841 (100)[c]
placental FN	3500 (47)	516 (61)

[a] Radioactivities associated with each FN band were estimated by a Fujix Bio-Imager Analyzer BA100 and expressed in an arbitrary unit. [b] Numbers in parentheses are relative radioactivities expressed in percent. [c] Radioactivities associated with cellular FN were taken as 100%.

had been elicited against the short peptide segment.

Immunoblot Analysis with Intact FNs. Binding specificities of the anti-peptide antibodies to the different forms of FNs were further examined by immunoblot analysis (Figure 3). By SDS–PAGE, plasma FN gave closely spaced doublet bands (Figure 3A, lane 1), of which only the upper rim of the larger subunit was immunostained with the anti-IIICS/Δ_2 antibodies (Figure 3B, lane 1). Thus, the IIICS/Δ_2 extra peptide appears to be included in a minor, but not in the major, subunit species of plasma FN, being consistent with the low binding capability of the antibodies with plasma FN adsorbed on the plastic surface (Figure 2B).

Unlike plasma FN, cellular and placental FNs gave a broad band on SDS–PAGE migrating slightly above plasma FN (Figure 3A, lanes 2 and 3). These bands were almost entirely immunostained with anti-IIICS/Δ_2 antibodies (Figure 3B, lanes 2 and 3). Densitometric analysis of the radioactivities associated with each protein-stained band showed that cellular FN bound nearly twice as many antibodies as placental FN (Table I), in agreement with the results obtained with the solid-phase binding analysis (Figure 2). A small difference in the antibody binding capability among three different FNs, observable between the immunoblot and solid-phase binding assays, could be due to a difference in the conformation of the proteins fixed on different solid supports. The binding of the anti-IIICS/Δ_2 antibodies to these FNs was specific to the IIICS/Δ_2 sequence, since immunostaining with the antibodies was completely abolished in the presence of the IIICS/Δ_2 peptide (data not shown).

The anti-ED-A antibodies displayed a clear binding specificity toward the three different FNs. Cellular and placental FNs were immunostained with the anti-ED-A antibodies, whereas plasma FN was completely devoid of the reactivity with the antibodies (Figure 3C, lanes 1–3). Quantitation of the radioactivities associated with each protein-stained band indicated that cellular FN bound significantly more antibodies than placental FN (Table I). Immunostaining with the antibodies was completely inhibited by the ED-A peptide, confirming the binding specificity of the antibodies (data not shown).

Immunoblot Analysis of the Cathepsin D Digests. Reactivities of the anti-peptide antibodies were further examined with cathepsin D digests of FNs. Limited proteolysis of plasma FN with cathepsin D has been shown to selectively cleave the protein at, or near, the junction between the "cell" and "heparin 2" domains, releasing the C-terminal "heparin 2" and "fibrin 2" domains of the two major subunits as M_r 70000 and 60000 fragments (Richter et al., 1981; Sekiguchi et al., 1985, 1986b). The fragments derived from the C-terminal region were specifically recognized by the monoclonal antibody FN8-12 (Figure 4A, lane 1), which is directed to the "fibrin 2" domain (M. Katayama and K. Sekiguchi, unpublished observation). The monoclonal antibody also immunostained a minor fragment with M_r 83000. Interestingly, the anti-IIICS/Δ_2 antibodies bound neither the M_r 70000 nor the M_r 60000 fragment but strongly immunostained the M_r 83000 fragment (Figure 4B, lane 1). These results indicate that the IIICS/Δ_2 extra peptide region was only present in a minor subunit of plasma FN, which gave rise to the C-terminal M_r 83000 fragment upon cathepsin D digestion. None of these or other cathepsin D fragments from plasma FN was recognized by the anti-ED-A antibodies (Figure 4C, lane 1), as expected from the inability of the antibodies to recognize intact plasma FN.

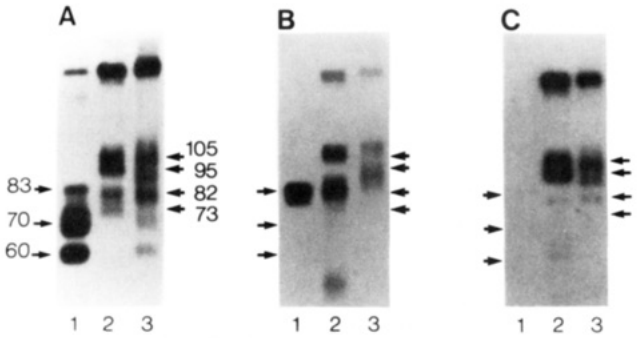

FIGURE 4: Immunoblot analysis of the cathepsin D digests of FNs with anti-peptide antibodies. FNs were digested with cathepsin D as described under Experimental Procedures. The cathepsin D digests (10 μg of protein) were separated on an 8% SDS–polyacrylamide gel in triplicate, transferred to a nitrocellulose membrane, and then immunostained with monoclonal antibody FN8-12 (panel A), anti-IIICS/Δ_2 antibodies (panel B), or anti-ED-A antibodies (panel C). Lane 1, plasma FN; lane 2, cellular FN; lane 3, placental FN. Relative molecular mass of the fragments from plasma and cellular FNs is indicated at the left and right, respectively, of panel A. The positions of these fragments are also indicated with arrows in panels B and C.

In contrast, the C-terminal region of cellular FN was released by cathepsin D as four major fragments with M_r 105000, 95000, 82000, and 73000 (Sekiguchi et al., 1986a), all of which were identified by immunostaining with the monoclonal antibody FN8-12 (Figure 4A, lane 2). The anti-IIICS/Δ_2 antibodies selectively recognized the M_r 105000 and 82000 fragments (Figure 4B, lane 2), whereas the anti-ED-A antibodies predominantly immunostained the M_r 105000 and 95000 fragments (Figure 4C, lane 2). These results indicate that the M_r 105000 fragment contained both the ED-A and IIICS/Δ_2 extra peptide regions; the M_r 95000 fragment contained the ED-A, but not the IIICS/Δ_2; the M_r 82000 fragment contained the IIICS/Δ_2, but not the ED-A; and the M_r 73000 fragment contained neither the ED-A nor the IIICS/Δ_2. Thus, all possible combinations of the presence or absence of these two extra peptide regions appear to exist within a single subunit polypeptide.

Cathepsin D digestion of placental FN yielded at least five discernible fragments with M_r 100000, 89000, 80000, 70000, and 60000, immunostained with the monoclonal antibody FN8-12 (Figure 4A, lane 3). FN8-12 also broadly immunostained the M_r 115000–110000 region. Among these fragments, the anti-IIICS/Δ_2 antibodies preferentially immunostained M_r 115000 and 89000 fragments (Figure 4B, lane 3), whereas the anti-ED-A antibodies recognized the M_r 100000 and, to a lesser extent, the M_r 115000–110000 and 89000 fragments (Figure 4C, lane 3). The M_r 70000 and 60000 fragments, comigrating with the major C-terminal fragments from plasma FN, were recognized neither by the anti-IIICS/Δ_2 nor by the anti-ED-A antibodies (Figure 4B, C, lane 3). These results suggest that placental FN is more heterogeneous than plasma and cellular FNs in the structure of the C-terminal region and consists of both the cellular-form subunits containing the ED-A and/or IIICS/Δ_2 extra peptide

regions and the plasma-form subunits lacking both of them.

Discussion

In the present study, the presence or absence of the two alternatively spliced peptide segments, ED-A and IIICS/Δ_2, in different FN isoforms was investigated with site-specific antibodies elicited by immunization with synthetic peptides. Since such anti-peptide antibodies may have preferential affinity and specificity for the epitopes existing as the free peptides or in the unfolded protein conformation rather than for those incorporated into the three-dimensional structure of the intact proteins, it appears important to examine the reactivity of the antibodies with reduced, SDS-denatured proteins preferably before and after proteolytic degradation. In fact, the anti-ED-A antibodies failed to recognize the intact cellular FN coated on the plastic surface but did bind to the same protein after reduction with 2-mercaptoethanol and denaturation with SDS with or without prior cathepsin D digestion. In contrast, plasma FN did not react with anti-ED-A antibodies under the same condition, verifying the absence of the ED-A extra peptide segment in plasma FN.

Despite extensive studies on the expression of the ED-A sequence in FNs at both the protein and mRNA levels (Kornblihtt et al., 1984a,b; Paul et al., 1986; Peters et al., 1986; Borsi et al., 1987; Vartio et al., 1987; Norton & Hynes, 1987), little has been known about the expression and regulation of the IIICS/Δ_2 sequence in human FNs. It has been assumed that the IIICS/Δ_2 extra peptide region may be absent in plasma FN (Umezawa et al., 1985; Garcia-Pardo et al., 1985; Sekiguchi et al., 1986a), but our present results clearly show that the IIICS/Δ_2 region is expressed not only in cellular and placental FNs but also in a minor subunit of plasma FN. This is in striking contrast to the exclusive expression of the ED-A peptide region in cellular, but not in plasma, FN (Paul et al., 1986; Peters et al., 1986; Borsi et al., 1987; Vartio et al., 1987; and this study). Thus, the IIICS/Δ_2 region cannot be a specific marker for distinguishing plasma FN from cellular and other forms of FNs.

A novel cell adhesive activity represented by a tetrapeptide Arg-Glu-Asp-Val (referred to as REDV in one-letter amino acid codes) was recently identified in the IIICS/Δ_2 sequence (Humphries et al., 1986, 1987). The REDV adhesion signal seems to be preferentially utilized by certain mouse melanoma cells, but not by fibroblasts. The presence of the IIICS/Δ_2-containing isoform(s) in plasma, though to a lesser extent, may well be consistent with the observation that human plasma FN could mediate the REDV-dependent adhesion of the mouse melanoma cells (Humphries et al., 1986), although a related activity was also identified in the N-terminal 25 residues of the 120 amino acid IIICS sequence (Humphries et al., 1987). Since the anti-IIICS/Δ_2 antibodies, unlike the anti-ED-A antibodies, are capable of recognizing nondenatured FNs in solution and those coated on the substrates or insolubilized in the extracellular matrix (K. Sekiguchi, unpublished observation), the antibodies should be useful for defining the biological function of the IIICS/Δ_2 region.

Recent studies on the alternative splicing of FN pre-mRNA at the IIICS region have shown that nearly 40% of the total FN mRNAs in human liver, the suggested source of plasma FN, are those containing the entire 360-base IIICS sequence (Oyama et al., 1989). It has not yet been well understood why the relative abundance of the IIICS/Δ_2-containing subunits in plasma FN is much less than that of the IIICS/Δ_2-containing FN mRNA in liver. It may be possible that the IIICS/Δ_2-containing isoforms are preferentially removed from circulating plasma by binding either to the blood vessel wall or to the connective tissue matrix. Deposition of plasma FN in the tissue matrices and basement membranes has been reported by Oh et al. (1981).

Mild cathepsin D digestion has been successfully applied to show the molecular heterogeneity of FNs at the C-terminal region (Richter et al., 1981; Sekiguchi et al., 1985, 1986b). Immunoblot analysis of the cathepsin D digests of cellular and placental FNs with anti-ED-A and anti-IIICS/Δ_2 antibodies indicates that all possible combinations of the presence or absence of these two extra peptides are indeed expressed in cellular and probably in placental FNs. These results strongly suggest that alternative splicing at the ED-A and IIICS region is regulated independently.

In contrast to plasma and cellular FNs, FNs in tissue matrices are difficult to isolate in a large quantity sufficient for conventional biochemical analysis (Bray et al., 1981). Placental FN is the only tissue FN successfully purified to homogeneity in a milligram quantity (Zhu et al., 1984; Isemura et al., 1984). Despite extensive characterization of the carbohydrate structure of placental FN (Zhu et al., 1984; Isemura et al., 1984), little has been known about the structure and isoform diversity of this FN. Our results strongly suggest that placental FN is more heterogeneous in the subunit composition than plasma and cellular FNs, consisting of both plasma- and cellular-type subunits. The cathepsin D fragments derived from the C-terminal region of placental FN were a mixture of those obtained from plasma FN and those containing the ED-A and/or IIICS/Δ_2 regions, which are characteristic of cellular FN. Furthermore, the relative abundance of the subunits containing the ED-A or IIICS/Δ_2 extra region in placental FN was much higher than in plasma FN, but significantly lower than in cellular FN. It is unlikely that the plasma-type subunits in placental FN were derived from contaminating blood, because the tissue was repeatedly washed, prior to extraction of FN, by homogenization in saline until the homogenate became almost colorless. This is further supported by the observation that the placental FNs purified from the second and third urea extracts of the same placenta yielded essentially the same cathepsin D fragments as those obtained from that purified from the first urea extract (K. Sekiguchi, unpublished observation).

Despite the importance of alternative pre-mRNA splicing in generating multiple FN isoforms, posttranslational modifications such as glycosylation, phosphorylation, and sulfation may also contribute to the heterogeneity of FN subunits. In particular, placental FN has been shown to contain polylactosamine carbohydrates (Zhu et al., 1984). The complex fragmentation pattern of the C-terminal region of placental FN by limited cathepsin D digestion could be partly due to the presence of such large carbohydrate units, although such carbohydrates have not yet been identified in the C-terminal region of placental FN.

Although placental FN is thus far the only tissue FN that can be purified in a relatively large quantity, it may be irrational to simply extend the observations obtained with placental FN to other tissue FNs. Our previous studies have indicated that FNs in human fetal lung predominantly consist of cellular-form FN, whereas the plasma-form FN accumulates in adult lung (Sekiguchi et al., 1986b). The results reported herein are consistent with those observations, since most, if not all, of the placental tissue used in this study was of fetal origin. It remains to be determined, however, whether FNs insolubilized in adult tissues other than lung are mostly the plasma form.

Recently, a third region for alternative splicing of FN pre-mRNA, termed ED-B, has been identified (Zardi et al., 1987; Schwarzbauer et al., 1987; Gutman & Kornblihtt, 1987). The overall complexity of FN isoforms should be, therefore, defined by a combination of the three alternatively spliced regions. The use of synthetic peptides as immunogens for eliciting site-specific antibodies, proved to be successful in the present study, should provide a means to study the expression of the ED-B extra region and its coordination with other alternatively spliced peptide segments.

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