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Functional domain structure of fibronectin

(thermolysin/gelatin binding/heparin binding/cell adhesion)

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ABSTRACT **Structural domains of fibronectin (FN) and their ability to associate with cell surface components have been systematically investigated. Plasma FN was cleaved into three structural domains (M_r 150,000–140,000, 40,000, and 32,000) by sequential digestion with trypsin and thermolysin. A single digestion with thermolysin alone generated M_r 150,000–140,000, 40,000, and smaller fragments. With the inclusion of thermolysin, but not with other proteases, one can, with a high yield, dissect FN simultaneously into three clearly distinctive functional domains. Of three major fragments, only the M_r 40,000 fragment bound to a gelatin column; this fragment contained essentially all of the carbohydrates present in the original FN. In contrast, heparin-binding sites were localized on both the M_r 150,000–140,000 and 32,000 fragments but not on the M_r 40,000 fragment. Only the M_r 150,000–140,000 fragments and intact FN promoted cell spreading, whereas the M_r 40,000 and 32,000 fragments could induce cell attachment but failed to promote cell spreading. These results indicate that FN is composed of (at least) three structural domains that are functionally distinct from each other.**

Fibronectin (FN), previously called "galactoprotein a" (1, 2) or "LETS" (3), is a major cell surface glycoprotein of fibroblasts and other cell types and is known to be markedly decreased after oncogenic transformation (1, 3–5). There is much evidence (3–6) indicating that it plays a role in cell–cell and cell–substratum adhesion, maintenance of cell morphology, and cell motility. It is also present in plasma (in which it is known as "cold-insoluble globulin"), and this plasma form has most, if not all, of the chemical and biological properties of its cellular counterpart (5, 7).

FN exists as a dimer or multimer linked through disulfide bonds (8–10). After reduction, it gives a single broad or closely spaced doublet bands on $NaDodSO_4$/polyacrylamide gel electrophoresis with M_r 210,000–230,000. Physicochemical studies indicate that it is composed of several domains connected by flexible polypeptide segments (11, 12). Such a domain structure has been also suggested by the results obtained from the proteolytic and chemical fragmentation of FN (2, 13).

FN exhibits a variety of affinities toward various substances, including collagen (14, 15), fibrin (16), glycosaminoglycan (17, 18), and possibly FN itself (18, 19). Because it is likely that these biological activities reside on certain structural domain(s), it is interesting to examine on which domain each diverse biological function is located. Recently, collagen-binding fragments have been isolated after hydrolysis with cathepsin D (20), trypsin (13, 21), or chymotrypsin (22, 23), but it was not clear whether the fragments had an affinity to other components. With the conditions described so far (13, 20–23), proteolysis was too extensive to obtain distinctive functional domains simultaneously in a high yield.

In this communication, we present a procedure for proteolytic fragmentation of plasma FN with trypsin and thermolysin or thermolysin alone; the intact molecules are cleaved into three distinct structural domains almost quantitatively. Only one of these domains exhibits gelatin-binding activity, the domain that contains essentially all the carbohydrates. The other two domains bind to heparin. The ability to promote cell attachment and spreading is localized in one of the glycosaminoglycan-binding domains.

MATERIALS AND METHODS

Materials. Thermolysin (protease, type X), gelatin (from swine skin, type I), and soybean trypsin inhibitor were obtained from Sigma; trypsin from Worthington; sialidase of *Vibrio cholerae* from Behring Diagnostics (Somerville, NJ); galactose oxidase from Kabi (Stockholm, Sweden); carrier-free $Na^{125}I$ and NaB^3H_4 from Amersham; heparin-agarose from Pierce; Sepharose 4B and Sephadex G-100 from Pharmacia; DEAE-cellulose (DE-32) from Whatman (Kent, England). Phosphoramidon was a generous gift from T. Aoyagi, Institute of Microbial Chemistry, Tokyo. Sialidase of *Arthrobacter ureafaecalis* was kindly donated by Masaki Saito, Institute of Gerontology, Tokyo. Gelatin-Sepharose 4B and soybean trypsin inhibitor-Sepharose 4B were prepared according to Cuatrecasas and Anfinsen (24). Proteins were iodinated by a chloramine-T method (25). Labeling of sugar moieties by sialidase/galactose oxidase treatment followed by NaB^3H_4 reduction was performed as described (26).

Isolation and Fragmentation of FN. FN was purified from hamster plasma by affinity chromatography on a gelatin-Sepharose 4B column as described (2) and extensively dialyzed against phosphate-buffered saline diluted 1:4 ($\frac{1}{4}$ × NaCl/P_i) containing 1 mM EDTA, pH 8.0. Digestion of intact FN (≈1 mg of protein per ml) with trypsin was performed at room temperature with trypsin at 1 μg/ml. After 15 min, the digests were rapidly passed through soybean trypsin inhibitor-Sepharose 4B to remove trypsin and then applied to gelatin-Sepharose 4B column (3–4 mg of gelatin per ml of packed gel). Unbound fractions were pooled, concentrated by vacuum dialysis, and partially purified on a Sephadex G-100 column. The bound fraction was eluted with NaCl/P_i buffer containing 4 M urea and dialyzed against $\frac{1}{4}$ × NaCl/P_i buffer containing 1 mM EDTA, pH 8.0, for three days at 4°C. Subsequent thermolysin digestion was performed as follows: Gelatin-bound tryptic fragments (0.5–1 mg of protein per ml) were diluted with an equal volume of 50 mM Tris·HCl (pH 7.6) containing 25 mM KCl and 10 mM $CaCl_2$ and incubated with thermolysin (2.5 μg/ml) at room temperature for 4 hr. The reaction was terminated by adding EDTA (10 mM) and the digests were applied to a gelatin-Sepharose column. Unbound and bound fragments were obtained as described above. Direct thermolysin digestion of intact molecules was performed in the same way as digestion of gelatin-bound tryptic fragments. Fragments of M_r 150,000–140,000 were further purified from gelatin-unbound

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Abbreviations: FN, fibronectin; NaCl/P_i buffer, 8.1 mM Na_2HPO_4/1.5 mM KH_2PO_4/137 mM NaCl/2.7 mM KCl (pH 7.4).

thermolysin fragments by subsequent DEAE-cellulose chromatography. Protein concentration was determined by the method of Lowry *et al.* (27).

Binding of FN and Its Fragments to Heparin-Agarose. To a heparin-agarose column (bed volume, 9–10 ml) equilibrated in 25 mM Tris·HCl (pH 7.6) containing 0.5 mM EDTA and 40 mM NaCl, 10 μl of ^{125}I-labeled FN or its fragments was applied after dilution with the Tris buffer to 1 ml. The column was washed with the Tris buffer and then adsorbed materials were eluted with the Tris buffer containing 0.5 M NaCl and 0.5 mM EDTA and then with the same buffer containing 8 M urea, 0.5 M NaCl, and 0.5 mM EDTA.

Cell Attachment Assay. NIL 2K cells were grown in Dulbecco's modified Eagle's medium plus 10% fetal calf serum as described (28). Confluent cells were harvested with 0.05% trypsin and 0.02% EDTA. After mixing with 2 vol of complete culture medium to inactivate trypsin, cells were washed three times with NaCl/P_i buffer containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ at 4°C and suspended in the same NaCl/P_i buffer. Wells of microtiter plates (Dynatech, Alexandria, VA) were incubated with 100 μl of intact FN or its fragments (5 μg/ml) at room temperature to coat the surface of wells with those proteins. After washing five times with 100 μl of NaCl/P_i buffer containing $CaCl_2$ and $MgCl_2$, 100 μl of cell suspension (5×10^5 cells per ml) prepared as described above was added to each well and incubated at 37°C for 1 hr. Unattached cells were removed by washing three times with 100 μl of NaCl/P_i buffer containing $CaCl_2$ and $MgCl_2$. The extent of cell attachment and spreading was examined under a phase-contrast microscope.

NaDodSO$_4$/Polyacrylamide Gel Electrophoresis. NaDodSO$_4$/polyacrylamide gel electrophoresis was performed according to Laemmli (29) in 8% polyacrylamide gels. Samples were reduced with 2% (vol/vol) 2-mercaptoethanol. The apparent molecular weight was estimated by using the following proteins as standards: skeletal muscle myosin, 200,000; β-galactosidase from *Escherichia coli*, 130,000; bovine serum albumin, 68,000; ovalbumin, 43,000; soybean trypsin inhibitor, 21,500; hemoglobin, 16,000. Fluorography of slab gels was performed according to Bonner and Lasky (30).

RESULTS

Separation of Structural Domains by Limited Proteolysis. Native FN migrates as closely spaced doublet bands with M_r 230,000 and 210,000 on NaDodSO$_4$/polyacrylamide gels under reducing conditions (Fig. 1, lane a). Mild trypsinization converted these to new doublet bands (M_r 200,000 and 180,000) and a fast-migrating band (M_r 32,000; Fig. 1, lane b). Only the doublet M_r 200,000–180,000 fragments bound to a gelatin-Sepharose column, leaving the M_r 32,000 fragment unbound (Fig. 1, lanes c and d). The M_r 32,000 fragment was further partially purified by Sephadex G-100 gel filtration (Fig. 1, lane e). The gelatin-bound M_r 200,000–180,000 fragments were further digested by thermolysin into two distinct fragments, one with M_r 150,000–140,000 and another with M_r 40,000 (Fig. 1, lane f). Only the M_r 40,000 fragment bound to the gelatin-Sepharose column; the doublet M_r 150,000–140,000 passed through (Fig. 1, lanes g and h). Interestingly, essentially all carbohydrate residues, labeled by the sialidase/galactose oxidase/NaB^{3}H$_4$ method, of original FN were recovered in the gelatin-bound M_r 40,000 fragment (Fig. 1, lane k; see *Discussion*).

Direct thermolysin digestion of intact FN generated three major fragments, the doublet M_r 150,000–140,000, the gelatin-binding M_r 40,000, and a M_r 24,000 fragment. In addition, smaller quantities of fragments with M_r 27,000 and 21,000 were also produced. When partially purified tryptic M_r 32,000 fragment was digested with thermolysin, the three fragments (M_r 27,000, 24,000, and 21,000) were produced and the M_r

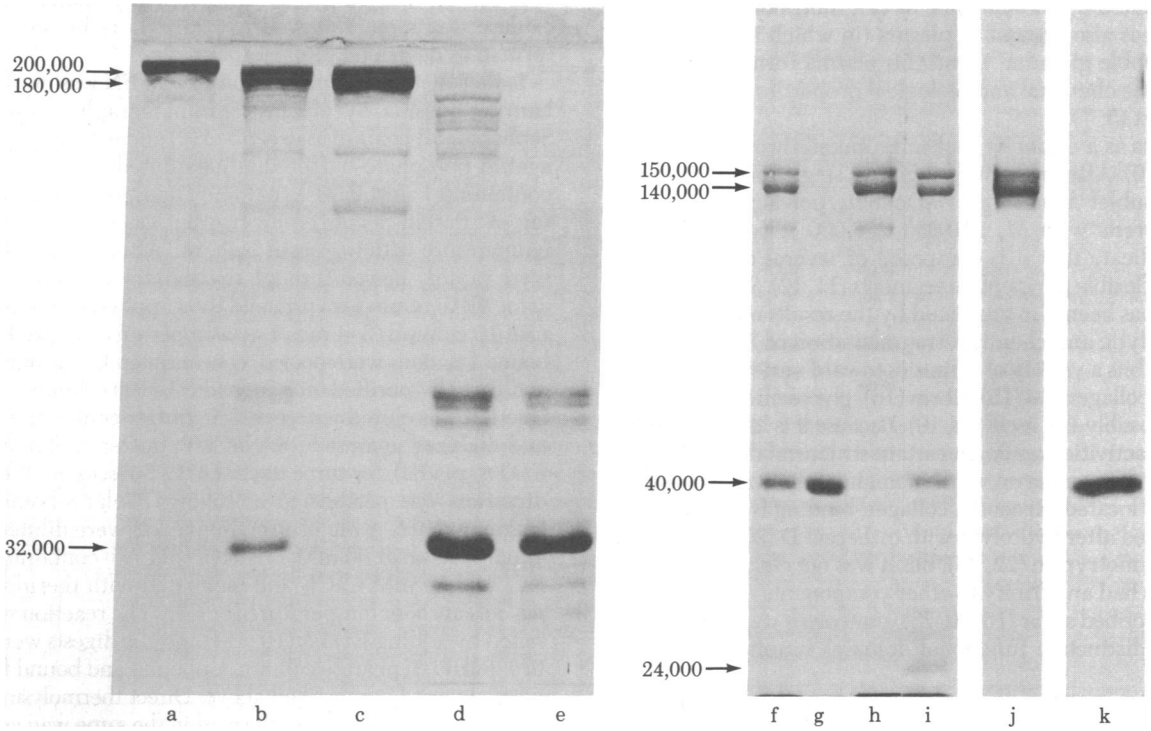

FIG. 1. NaDodSO$_4$/polyacrylamide gel electrophoresis of the products from sequential digestion of plasma FN with trypsin and thermolysin. Lane a, intact hamster plasma FN; lane b, whole trypsin digest of FN; lane c, gelatin-bound tryptic fragments; lane d, gelatin-unbound tryptic fragments; lane e, partially purified M_r 32,000 fragment; lane f, thermolysin digest of gelatin-bound tryptic fragments; lane g, gelatin-bound fragment of the thermolysin digest shown in lane f; lane h, gelatin-unbound fragments of the thermolysin digest shown in lane f; lane i, thermolysin digest of intact FN; lane j, purified M_r 150,000–140,000 fragments; lane k, fluorogram of thermolysin digest of the carbohydrate-labeled FN. The protein staining pattern of this gel was essentially like that of lane i.

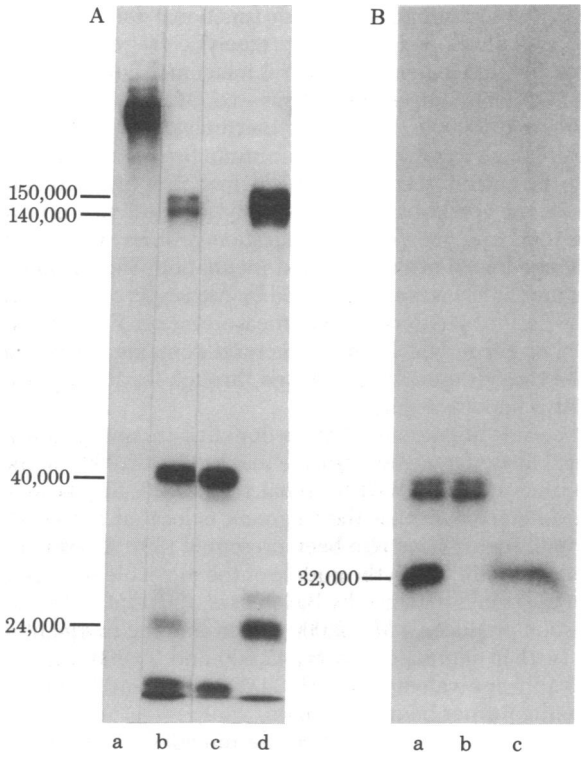

FIG. 2 (*A*) Identification of heparin-binding fragments. ^{125}I-Labeled FN was digested with thermolysin (2.5 μg/ml) at room temperature for 3 hr. After addition of phosphoramidon (5 μg/ml), a specific inhibitor of thermolysin, the digest was applied to a heparin-agarose column. Bound materials were eluted with 25 mM Tris buffer (pH 7.6) containing 4 M urea, 0.5 M NaCl, and 0.5 mM EDTA. Unbound and bound fractions were separately pooled, concentrated by vacuum dialysis, and analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis and subsequent autoradiography. Lane a, intact FN; lane b, thermolysin digest of FN; lane c, heparin-unbound fragments; lane d, heparin-bound fragments. The relative intensity of each band of gel b as observed by protein staining with Coomassie blue is considered to be essentially identical to that of lane i in Fig. 1, because thermolysin digestion was carried out at the same enzyme concentration. The decreased intensity of the M_r 150,000–140,000 bands is, therefore, possibly due to lower specific activity (expressed as cpm per mg of protein) of these fragments when compared with other fragments. This possibility was supported by the observation that the specific activity of the M_r 150,000–140,000 band was about one-fifth that of the M_r 40,000 and 32,000 bands when each purified fragment was separately labeled with Na^{125}I under the same conditions. (*B*) Gel electrophoretic analysis of bound and unbound fractions of heparin-agarose chromatography of partially purified M_r 32,000 fragment. Bound and unbound fractions underlined in Fig. 3*D* were separately pooled, concentrated, and analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis. Lane a, partially purified M_r 32,000 fragment; lane b, heparin-agarose column-unbound fragments; lane c, heparin-agarose column-bound fragment.

24,000 fragment was predominant among them (data not shown). The result indicates that these smaller fragments were derived from the M_r 32,000 domain. The pattern of the fragments obtained by direct thermolysin digestion of intact FN was almost unchanged during prolonged incubation (from 20 min to 4 hr; data not shown). Purification of the doublet M_r 150,000–140,000 could be accomplished by subsequent DEAE-cellulose chromatography (Fig. 1, lane j).

Identification of Heparin-Binding Fragments. Because plasma FN has been shown to interact with heparin (17, 31), we examined which fragments bound to a heparin-agarose column. When the whole thermolysin digest of intact FN was applied to a heparin-agarose column, both the M_r 150,000–

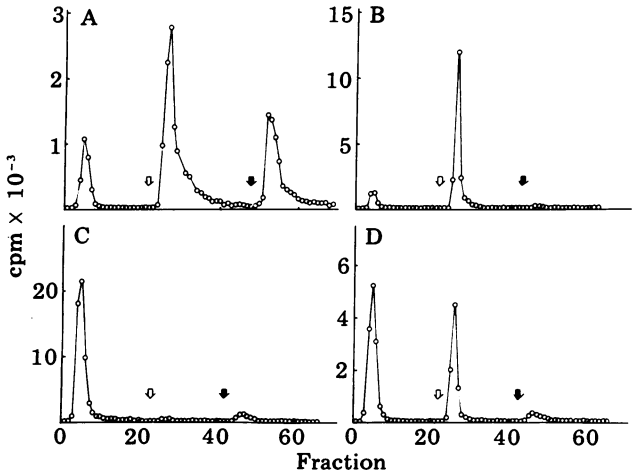

FIG. 3. Binding of purified thermolysin fragments to heparin-agarose. Iodinated purified fragments were applied to a heparin-agarose column and their elution profiles were compared. Open and closed arrows indicate the starting positions of elution with 0.5 M NaCl and 8 M urea/0.5 M NaCl, respectively. (*A*) Intact FN; (*B*) M_r 150,000–140,000 fragments; (*C*) M_r 40,000 fragment; (*D*) partially purified M_r 32,000 fragment.

140,000 and the M_r 24,000 fragments bound to the column, whereas the M_r 40,000 fragment failed to bind (Fig. 2*A*). To confirm this finding, binding to the heparin-agarose column by purified fragments as well as intact FN was examined. As expected, most of the intact FN and the M_r 150,000–140,000 fragments and also about 45% of the partially purified M_r 32,000 fraction bound to the column, although M_r 40,000 failed to bind (Fig. 3). Because the M_r 32,000 fraction used in these experiments contained some contaminants (see Fig. 1, lane e), we analyzed both the unbound and bound fractions from the heparin-agarose column (see Fig. 3*D*) by gel electrophoresis. As shown in Fig. 2*B*, M_r 32,000 was recovered only in the bound fraction, confirming that M_r 32,000 as well as M_r 150,000–140,000 has an ability to bind to heparin. A part of intact FN showed stronger interaction with the heparin-agarose column and was eluted only with addition of 8 M urea. The basis of this phenomenon is not known.

Identification of Fragments Responsible for Cell Attachment and Spreading. FN has been shown to promote cell attachment and spreading on plastic and glass surfaces (6). To identify the active domain that promotes this process, we examined the extent of cell attachment and spreading on microtiter plates that were coated with intact FN or its fragments. When plates were coated with intact molecules, extensive cell attachment could be seen and almost all of attached cells underwent spreading (Fig. 4*A*). Similar or slightly higher cell attachment and subsequent spreading could be also observed on the plates coated with M_r 150,000–140,000 fragments (Fig. 4*B*). When plates were coated with M_r 40,000 fragment, however, cell spreading was almost completely inhibited, although the degree of cell attachment was only slightly decreased (Fig. 4*C*). Similarly, purified M_r 32,000 fragment* mediated significant cell attachment but its ability to induce cell spreading was extremely low when compared with intact FN or M_r 150,000–140,000 fragments.

Because it is reasonable to assume that the promotion of cell attachment and spreading by each fragment depended on the

* To eliminate the influence of contaminant components in the partially purified M_r 32,000 preparation, M_r 32,000 fragment further purified on a heparin-agarose column was used in this experiment.

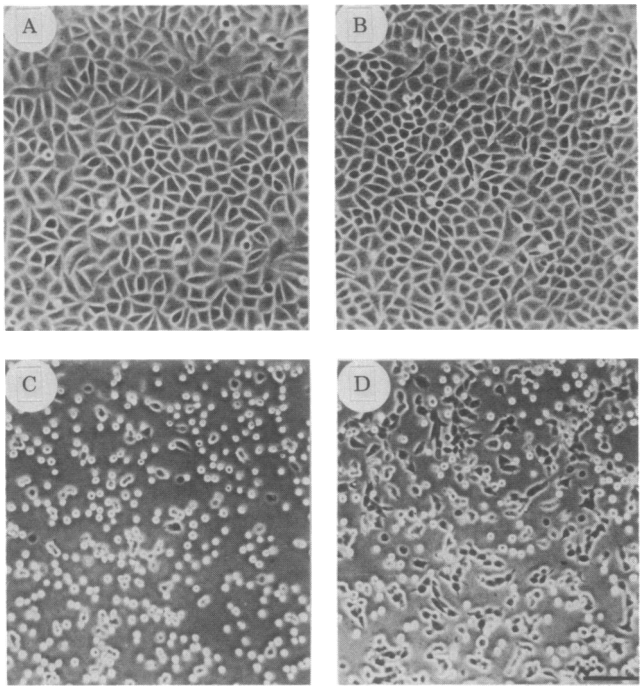

FIG. 4. Attachment of cells on plastic plates coated with intact FN and its fragments. Cells were incubated at 37°C for 1 hr on microtiter plastic plates precoated with various proteins (5 μg/ml) as indicated. Phase-contrast microscopy; bar = 100 μm. (*A*) Intact FN; (*B*) M_r 150,000–140,000 fragments; (*C*) M_r 40,000 fragment; (*D*) purified M_r 32,000 fragment.

amount of the fragment adsorbed on the plates, we measured the binding of intact FN and its fragments to the plastic plates. As shown in Table 1, there was virtually no significant correlation of these proteins between their adsorption efficiency and cell-spreading activity. This result indicates that the failure of M_r 40,000 and 32,000 fragments to promote cell spreading is not due to the lowered adsorption onto the plates but rather is due to the lack of specific interaction with the cell surface, which is a prerequisite for cell spreading after initial attachment.

DISCUSSION

One approach to understanding the structure–function correlation of such high molecular weight proteins as FN is to dissect intact molecules into several large fragments that retain the biological activities of the parent molecules. Such an approach was partially successful in isolating gelatin- (or collagen-) binding fragments after proteolysis (13, 20–23), although the proteolytic fragmentations under the conditions in those studies have been too extensive to preserve other functional domains or too slight to distinguish the basic functional domains. In this paper, we developed an ideal proteolytic fragmentation of plasma FN that sequentially cleaves intact molecules into three large fragments with clear functions—i.e., M_r 150,000–140,000, 40,000, and 32,000. The use of thermolysin was the key to separate these fragments with minimum further degradation. These fragments seem to form distinct structural domains, because the conditions of the proteolytic digestion employed were mild and the resulting digestion profiles were almost unchanged even after prolonged incubation. Such a domain structure for FN was also proposed by Alexander *et al.* (11, 12), on the basis of physicochemical measurements. Fig. 5 shows a model of FN in which three structural domains, represented by the three fragments, are linked through flexible protease-sensitive peptide segments.

Table 1. Adsorption of FN and its fragments on plastic plates

Additions	Proteins adsorbed,* % 1 hr	10 hr
Intact FN	46	82
M_r 150,000–140,000 fragments	38	59
M_r 40,000 fragment	41	52
M_r 32,000 fragment	50	88

Iodinated FN or its fragments (5 μg/ml) were incubated on microtiter plates at room temperature for 1 or 10 hr. After extensive washing, bound materials were solubilized with 1% $NaDodSO_4$ and 0.5 M NaOH.

* Average of four determinations.

The exact alignments of these domains are not yet known. Several lines of evidence indicate that the M_r 32,000 fragment is located at the COOH-terminal region (20, 32); however, contradictory data that the fragment is located at the NH_2-terminal region have also been presented (33). A possible location of M_r 40,000 in the middle of the molecule is suggested from the results obtained by Balian *et al.* (20) that cathepsin D digestion produced a M_r 72,000 gelatin-binding fragment that was further degraded into M_r 42,000 and 30,000 fragments (probably equivalent to our M_r 32,000 fragment) by plasmin. Only the former bound to gelatin.

Essentially all the carbohydrate moieties labeled by sialidase/galactose oxidase/NaB^3H_4 are localized on the M_r 40,000 fragment. Only through dissection with thermolysin can one demonstrate a clear localization of the carbohydrate in the M_r 40,000 fragment. With chemical dissection (*S*-cyanylation) we have demonstrated (2) a clustering of carbohydrate at the middle section of FN. Coincidence of the carbohydrate at-

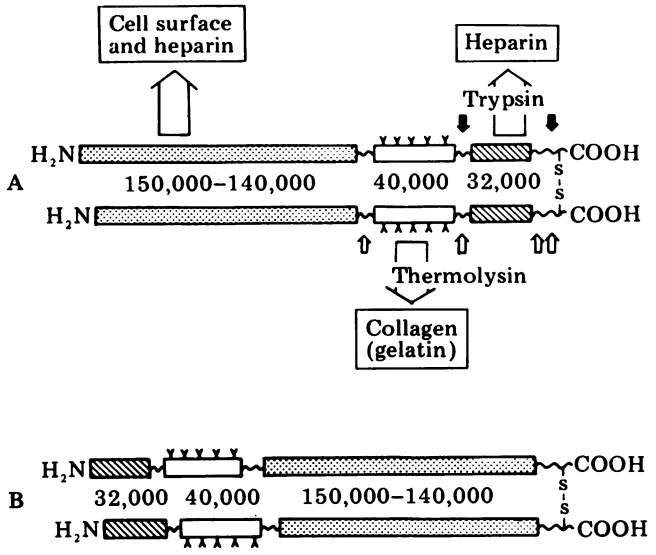

FIG. 5. A model for the functional domain structure of FN. Closed and open arrows indicate the susceptible sites for trypsin and thermolysin, respectively. Y represents a carbohydrate moiety attached to polypeptide chains. The number of carbohydrate chains was tentatively chosen according to Fukuda and Hakomori (2). Two possible alignments are shown; the upper (*A*) and the lower (*B*) models are different in the location of M_r 150,000–140,000 and 32,000 at either the COOH- or the NH_2-terminal region. In both models, the sequential alignment of the domain from either NH_2-terminal or COOH-terminal is identical. The model also explains the origin of the doublets seen in the reduced intact FN (M_r 230,000–210,000) and in the tryptic fragment (M_r 200,000–180,000): they were derived from the two different sizes of cell surface-binding domains (M_r 150,000–140,000).

tachment sites and the collagen-binding fragment suggested that the carbohydrate may have some role in collagen-binding activity. However, the experimental result that ^{125}I-labeled M_r 40,000 fragment bound to the gelatin column equally well after extensive digestion with *Arthrobacter* sialidase (which has a broader substrate specificity) followed by β-galactosidase, β-*N*-acetylhexosaminidase, and α-mannosidase clearly eliminated this possibility.

The most important feature of the domain structure of FN is that each domain exhibits different mutually exclusive biological activities. Gelatin-binding activity is localized on the M_r 40,000 fragment, whereas heparin-binding sites are on the other two domains. Activity in promoting cell spreading is localized on the M_r 150,000–140,000 fragments. Because FN constitutes an extracellular fibrillar matrix together with several types of collagen, glycosaminoglycan, and other components, such a functional domain structure offers a molecular basis for elaboration of the extracellular matrix. The associations of collagen (14, 34) and sulfated proteoglycan (18) with FN on the cell surface has been already described.

The distinct localization of active sites for cell spreading and gelatin binding was reported by Ruoslahti and Hayman (35) and more recently by Hahn and Yamada (23). They found an activity in promoting cell attachment and spreading in gelatin-unbound fragments. Hahn and Yamada fractionated gelatin-unbound fragments obtained by chymotryptic digestion with a Sephacryl S-300 column and found a strong activity in promoting cell spreading in a fragment with $M_r \approx 160{,}000$, which may correspond to our M_r 150,000–140,000 fragments. However, the proteolytic conditions they employed were so strong that it was difficult to distinguish original domains from their further degradation products—i.e., regions present in smaller fragments overlapped with those present in larger fragments. Thus, it is hard to elucidate how many structural domains are present in FN or how various biological functions of FN are expressed through each domain.

Although the mechanism of FN-mediated cell spreading is not yet well understood, it is believed that there is a specific receptor on the cell surface for substratum-adsorbed FN (6). Culp and his coworkers proposed a model of cell adhesion in which glycosaminoglycans play a critical role as a crosslinker between cell surface and substratum-adsorbed FN (36). Close association of externally added FN with glycosaminoglycans was recently demonstrated by Perkins *et al.* (18), using crosslinking reagents. However, the failure of the M_r 32,000 fragment, which bound to heparin, to promote cell spreading suggests that interaction between glycosaminoglycan and FN is not a prerequisite for cell spreading. Although M_r 150,000–140,000 fragments not only promote cell spreading but also bind to heparin, it seems possible that active sites for cell spreading and glycosaminoglycan binding reside on different portions of these fragments. Indeed, a strong activity in promoting cell spreading could be found in minor tryptic fragments that bound to neither gelatin nor heparin (preliminary observation), suggesting that M_r 150,000–140,000 fragments may be further divided into at least two functional subdomains, one responsible for cell spreading and another responsible for glycosaminoglycan binding.

Another possible candidate for a "cell adhesion receptor" may be cell surface FN itself, but so far there is no firm evidence demonstrating self-association of FN. The presence of other membrane proteins that could function as FN receptors in cell spreading is also possible.

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