# Release of Biological Activities from Quiescent Fibronectin by a Conformational Change and Limited Proteolysis by Matrix Metalloproteinases

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ABSTRACT: We reported that specific biological activities are confined to three domains of the fibronectin (Fn) molecule [Fukai et al. (1991) *J. Biol. Chem. 266*, 8807; Fukai et al. (1993) *Biochemistry 32*, 5746]: the potent ability to stimulate the adipocyte differentiation of ST-13 cells is in the amino-terminal fibrin-binding (Fib 1) domain (referred to as Fib 1 domain activity); the RGD-dependent activities that stimulate NIH-L13 cell migration and inhibit adiopcyte differentiation are in the central cell-binding (Cell) domain (Cell domain activity); and the activity that stimulats cell migration in a RGD-independent manner is in the carboxyl-terminal fibrin-binding (Fib 2) domain (Fib 2 domain activity). Human plasma Fn which was purified without exposure to a denaturant, such as urea, exhibited no Fib 1, Fib 2, or Cell domain activity. By exposure to urea or surface adsorption, Fn showed Cell domain activity but not those of the Fib 1 and Fib 2 domains. Whether the cryptic domain activities are disclosed or not depended on whether or not the responsible domains were irreversibly exposed from confined environments of Fn structure as confirmed by light-scattering measurement and enzyme immunoassay using domain-specific monoclonal antibodies. We then investigated the action of matrix metalloproteinases (MMPs) in liberating the Fib 1, Cell, and Fib 2 domain activities. Matrilysin released only the Cell domain activity. In contrast, stromelysin, collagenase, and especially gelatinase A additionally liberated the Fib 1 and Fib 2 domain activities. The Fib 1, Fib 2, and Cell domains acquired much higher activities when they were freed from linkage with adjacent domains. The results suggested that the Fib 1, Cell, and Fib 2 domains are buried in the native Fn structure, but are disclosed via two separate routes: the Cell domain activity is exposed by a conformational change and the others by proteolytic fragmentation.

Fibronectin (Fn)[1] is a cell adhesive glycoprotein present in blood plasma as a soluble form and in the extracellular matrix (ECM) as an insoluble fibrillar form (Hynes, 1989a; Yamada, 1989). The functional domains of Fn serve as specific binding sites for a number of different biological structures such as cell-surface integrins, collagen, fibrin, and glycosaminoglycans. These binding characteristics of the functional domains are important for allowing cells to adhere to ECM and consequently to modulate cell growth, migration, and differentiation.

On the other hand, a number of studies have indicated that some functional domains harbor various biological activities not detected with intact Fn (Humphries & Ayad, 1983; Czop et al., 1985; Homandberg et al., 1986, 1992; Clark et al., 1988; Werb et al., 1989). We also showed that unique biological activities are confined to the restricted three functional domains (Fukai et al., 1991, 1993a): a potent activity to induce adipocyte differentiation of ST-13 preadipocytes is in the amino-terminal fibrin-binding (Fib 1) domain; the central cell-binding (Cell) domain can either promote RGD (Arg-Gly-Asp)-dependent chemotactic migration of NIH-L13 fibroblasts or inhibit adipocyte differentiation of ST-13 cells; and the carboxyl-terminal fibrin-binding (Fib 2) domain can stimulate RGD-independent chemotactic migration of NIH-L13 fibroblasts at extremely low concentrations.

Two molecular processes that disclose these cryptic activities are presumed. First, these cryptic activities may be exposed through a conformational change of Fn. Several studies have demonstrated (Colonna et al., 1978; Alexander et al., 1979; Engel et al., 1981; Odermatt et al., 1982; Williams et al., 1982; Tooney et al., 1982; Erickson & Carrell, 1983; Rocco et al., 1983; Markovic et al., 1983; Welsh et al., 1983; Homandberg, 1987) that some functional domains are buried in the Fn structure and can be exposed by denaturation, by surface adsorption, or by binding with gelatin and heparin. However, little is known whether such changes in Fn structure actually liberate the biological activities buried in the Fn molecule. Especially, there has been little information about cryptic activities other than the cell adhesive function of Fn. Second, the activities may be liberated by proteolytic fragmentation of intact Fn. Fn is susceptible to proteolysis (Yamada, 1989), and indeed Fn fragments have been found in body fluids including inflam-

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[1] Abbreviations: Fn, fibronectin; Fib 1, amino-terminal fibrin-binding; Gel, gelatin-binding; Cell, central cell-binding; Hep, carboxy-terminal heparin-binding; Fib 2, carboxyl-terminal fibrin-binding; ECM, extracellular matrix; mAb, monoclonal antibody; MMP, matrix metalloproteinase.

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matory lesions (Clemmensen et al., 1983; Vartio et al., 1983; Carsons, 1987; Katayama et al., 1993). In this situation, the mode of proteolysis that releases the responsible domains without loss of their activities should be studied. Thus, the question as to how the biological activities buried in Fn molecule are liberated has not been fully answered.

During our investigation, we found that plasma Fn purified under mild experimental conditions without exposure to denaturant does not show the activities attributed to Fib 1, Cell, and Fib 2 domains. This quiescent Fn is useful for studying how these activities of Fib 1, Cell, and Fib 2 domains are buried in the intact Fn structure and how they are disclosed. Here we characterized the quiescent Fn molecule in terms of the correlation between molecular dimension and biological activity. We then studied activation of the quiescent Fn molecule by a conformational change and by proteolytic fragmentation. For the latter, we studied the action of matrix metalloproteinases (MMPs), since many investigations have indicated the involvement of this family of proteinases in various biological processes such as embryonic development, tissue remodeling, inflammation, tissue repair, and metastasis (Liotta et al., 1980). The results showed that gelatinase A releases the Fib 1, Cell, and Fib 2 domain activities.

## MATERIALS AND METHODS

*Materials.* Thermolysin (protease type X from *Bacillus thermoproteolyticus rokko*) and cathepsin D (bovine spleen) were purchased from Sigma Chemical Co. Four matrix metalloproteinases (MMPs) were purified in proenzyme forms as reported: gelatinase A (MMP2; EC 3.4.24.24) from the human glioblastoma cell line T98G (Miyazaki et al., 1993), matrilysin (MMP9; EC 3.4.24.23) from the human rectal carcinoma cell line CaR-1 (Miyazaki et al., 1990), interstitial collagenase (MMP1; EC 3.4.24.7) from the human hepatoma cell line HLE (Umenishi et al., 1991), and stromelysin (MMP3; EC 3.4.24.17) from the rat transformed cell line RSV-BRL (Umenishi et al., 1990). Rabbit polyclonal antibodies against rat plasma Fn were raised as described (Fukai et al., 1991). Monoclonal antibodies (mAbs), FN9-1, FNC4-4, FN12-8 or FN30-8, FNH3-8, and FN8-12, which specifically recognize Fib 1, gelatin-binding (Gel), Cell, carboxyl-terminal heparin-binding (Hep), and Fib 2 domains, respectively (see Figure 2), were purchased from Takara Biomedicals (Tokyo). Domain-specific polyclonal antibodies were kindly provided by Dr. Kiyotoshi Sekiguchi, Director of the Research Institute, Osaka Medical Center for Maternal and Child Health.

*Intact Fn Preparations and Their Treatment To Induce a Conformational Change.* Intact Fn was purified from human plasma using either a gelatin affinity gel or a heparinoid affinity material (sulfated cellulofine) as follows.

*(A) Purification Using a Gelatin Affinity Matrix.* Affinity chromatography using a gelatin−Sepharose column was performed as described by Engvall and Ruoslahti (1977), in which Fn bound to the gel was eluted with buffer A (50 mM NaCl, 50 mM Tris-HCl buffer, pH 7.3) containing 4 M urea and the proteinase inhibitors. Intact Fn thus purified is referred to as *u*-Fn.

*(B) Purification Using Sulfated Cellulofine.* Human plasma (50 mL) was gel-filtered through a Sephacryl S-300 column (5 × 90 cm) equilibrated with buffer A. Fractions containing Fn were applied to a column (2.6 × 15 cm) of sulfated cellulofine (Seikagaku Kogyo, Tokyo), washed exhaustively with buffer A containing 0.2 M NaCl (about 1500 mL of the buffer), and then eluted with a linear gradient of NaCl (0.2−1 M). The Fn peak eluted at around 0.5 M NaCl was pooled and gel-filtered through a Sephacryl S-300 column equilibrated with buffer A containing 0.3 M NaCl. Fractions containing Fn were pooled and dialyzed thoroughly against PBS(−). About 3−5 mg of purified Fn was finally recovered. This Fn is referred to as *s*-Fn.

The *u*- and *s*-Fns were denatured by dialysis against buffer A containing 4 M or 8 M urea or 6 M guanidine hydrochloride at 25 °C for 18 h. The dialysis buffer was then changed to PBS(−) to remove the denaturant.

The *s*-Fn (50 μg/mL) was incubated at 37 °C for 20 min with heparin (25 μg/mL) or gelatin (30 or 50 μg/mL), and the incubation mixture was then coated onto culture dishes. Alternatively, after diluting to the appropriate concentrations of Fn, the incubation mixture was directly assayed.

*Fn Fragments.* A 24-kDa fragment originating from the Fib 1 domain (24K Fib 1 fragment), a 50-kDa fragment from the Gel domain (50K Gel fragment), a 30-kDa fragment from the Hep domain (30K Hep fragment), and a 21-kDa fragment from the Fib 2 domain (21K Fib 2 fragment) were purified from the thermolysin digest of *u*-Fn as described (Fukai et al., 1991). A 67-kDa fragment containing the Fib 1 and Gel domains (67K Fib 1 fragment) and a 150-kDa fragment containing the Hep 2 and Fib 2 domains dimerized by disulfide bond(s) were isolated from the cathepsin D digest of Fn as described (Richter et al., 1981). The latter fragment was used as a 70-kDa fragment after reduction and methylation with iodoacetamide (70K Fib 2 fragment).

*Evaluation of Tertiary Structure of Fn Molecules.* The molecular dimensions of Fn were estimated by measuring dynamic light scattering using a DLS-700 from Otsuka Electronics with an $Ar^+$ laser (488 nm) at 25 °C and a scattering angle of 35 °C. Each Fn preparation in 20 mM Tris-HCl buffer containing 0.15 M NaCl (pH 7.4) was measured. The diffusion coefficient was estimated by analyzing the measured intensity autocorrelation function in the homodyne mode by the cumulant method (Chu, 1991). The diffusion coefficient value for Fn was confirmed to be independent of the protein concentration in the range from $1.7 \times 10^{-5}$ to $8.3 \times 10^{-5}$ M and a scattering angle from 20 to 90°. The Stokes radius was calculated from the Stokes−Einstein equation (Pusey & Tough, 1985).

The arrangement of Fib 1, Cell, and Fib 2 domains in the Fn molecule was presumed by measuring the immunoreactivity of the different Fn preparations toward the mAbs that recognized the corresponding domains of Fn. A sandwich-type enzyme immunoassay was performed, in which the domain-specific mAb was coated onto 96-well microtiter plates as a primary antibody, and Fn or Fn fragments were sandwiched by the biotinylated anti-Fn polyclonal antibody.

*Degradation of Fn by MMPs.* Degradation by MMPs was carried out (Miyazaki et al., 1990; Umenishi et al., 1990) as follows. The purified latent forms of MMPs were activated by incubating with 1 mM *p*-aminophenylmercuric acetate at 37 °C for 1 h. The *s*-Fn in buffer A was mixed with each of the activated forms (1/50 w/w); then the reaction was started by adding 2 mM $CaCl_2$. After incubation at 37 °C for 24 h, the reaction was stopped by heating at 70 °C for 10 min in the presence of 10 mM 1,10-*o*-phenanthroline.

*Evaluation of Fib 1 Domain Activity.* The Fib 1 domain activity was assessed by estimating the ability to induce the adipocyte differentiation of ST-13 cells as described earlier (Fukai et al., 1993a). Briefly, ST-13 preadipocytes were seeded onto 24-well culture dishes that were coated with or without 50 μg/mL *s*- or *u*-Fn and then cultured with medium containing insulin (induction medium) in the absence or presence of the sample. The number of differentiated adipocytes was counted daily in 10 random fields (×200). Cytosolic glycerophosphate dehydrogenase (EC 1.1.1.8) activity of ST-13 cells, a marker enzyme of the differentiation, was also determined after 10 days of culture.

*Chemotaxis Assay and Evaluation of Fib 2 Domain Activity.* Chemotaxis was assayed in blind-well chambers using a PVP-free polycarbonate filter (8 μm pore size, Nucleopore) as described (Fukai et al., 1993b). Samples were either placed in the lower compartment of the chamber or coated on the lower surface of the filter as described by Makabe et al. (1990).

The 21K Fib 2 fragment promotes the RGD-independent chemotactic migration of NIH-L13 fibroblasts at extremely low concentrations (below 5 nM) where intact Fn does not exhibit the activity (Fukai et al., 1991). To evaluate the Fib 2 domain activity, the chemotactic activity was assayed at attractant concentrations ranging from 0.05 to 500 nM. The Fib 2 domain activity is represented as the number of migrated cells at 0.5 nM.

## RESULTS

*Quiescent Fn and Its Activation through Conformational Change.* We demonstrated that the RGD-dependent activities that stimulate NIH-L13 cell migration and inhibit adipocyte differentiation of ST-13 cells are localized in the Cell domain (referred to as Cell domain activity), that the potent ability to induce adipocyte differentiation is in the Fib 1 domain (Fib 1 domain activity), and that the activity to stimulate NIH-L13 fibroblast migration at extremely low concentrations below 5 nM is in the Fib 2 domain (Fib 2 domain activity).

Intact Fn purified by the standard method (*u*-Fn) showed chemotactic activity toward NIH-L13 fibroblasts that was competed by a peptide containing the cell adhesive RGD sequence (Figure 1A). The *u*-Fn also showed another Cell domain activity (inhibition of the adipocyte differentiation of ST-13 cells) (Table 1). When Fn was purified under mild conditions without exposure to denaturants such as urea, *s*-Fn hardly showed one of the two Cell domain activities, namely, chemotactic activity (Figure 1B). By exposure to 4 M urea, Fn acquired Cell domain activity. That is, *s*-Fn stimulated cell migration in an RGD-dependent manner, and its chemotactic dose−response profile closely resembled that of *u*-Fn (Figure 1B). The *s*-Fn also acquired Cell domain activity after coating on the lower surface of the membrane used for the chemotaxis assay (Figure 1B), suggesting that an RGD-dependent active region in the Cell domain was disclosed by surface adsorption. Supporting this assumption, *s*-Fn coated on the culture dishes also showed another Cell domain activity: the insulin-induced adipocyte differentiation of ST-13 cells was inhibited when they were cultured on the dish coated with *s*-Fn (Table 1).

Typical Fib 1 and Fib 2 domain activities as observed with the isolated 24K Fib 1 and 21K Fib 2 fragments are shown in Table 1 and Figure 1A, respectively. The Fib 2 domain activity represented as the number of migrated cells at 0.5 nM of each sample is summarized in Table 2. These activities were undetectable in *u*-Fn, and also treating *s*-Fn with a higher concentration of urea (8 M) and guanidine hydrochloride (6 M) failed to disclose these Fib 1 and Fib 2 domain activities (Tables 1 and 2). It can therefore be presumed that once exposed, Fib 1 and Fib 2 domains are buried again after removal of the denaturant. We studied if interaction of *s*-Fn with heparin and gelatin (type I) could disclose the Fib 1 and Fib 2 domain activities. With respect to the Fib 1 domain activity, ST-13 cells were cultured on dishes coated with mixed solutions of *s*-Fn and heparin or gelatin. However, there was no changing the differentiation inhibitory activity of the Fn (Table 1). Similarly, incubation of heparin or gelatin with *s*-Fn did not elicit the chemotactic activity at the lower concentration (Table 2). Similar results were obtained using *s*-Fn (data not shown).

*Structural Features of u- and s-Fns.* We assessed the difference between *u*- and *s*-Fn molecules in terms of structural features. The molecular dimensions of Fn were estimated by dynamic light-scattering measurement. The diffusion coefficient of *u*-Fn was estimated to be $1.759 \times 10^7$ cm$^2$/s. In contrast, *s*-Fn represented a considerably higher

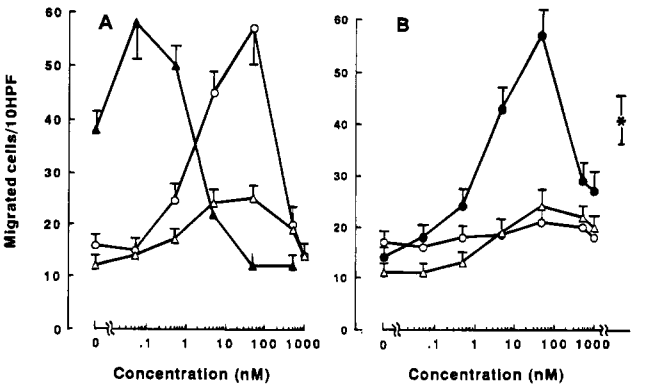


FIGURE 1: Chemotactic migration of NIH-L13 fibroblasts in response to *u*- and *s*-Fns. (A) *u*-Fn (○); *u*-Fn mixed with GRGDSP peptide (0.2 mg/mL) (△); 21K Fib 2 fragment (▲). (B) *s*-Fn (○); *s*-Fn treated with 4 M urea (●); urea-treated *s*-Fn mixed with GRGDSP (△). Another assay was performed using the filter coated with 50 μg/mL *s*-Fn (asterisk). Data represent the mean numbers of migrated cells per 10 high-powered fields (HPF) (×200) ± SE of three determinants.

Table 1: Effects of *s*-Fn and *s*-Fn Treated with Denaturant on Adipocyte Differentiation of ST-13 Cells[a]

| | no. of adipocytes/10HPF | GPD activity [μmol min$^{-1}$ (mg of protein)$^{-1}$] |
|---|---|---|
| induction medium (control) | 63 ± 26 (100) | 12.5 ± 1.1 (100) |
| +24K Fib 1 fragment[c] | 671 ± 111 (1065)[b] | 175.3 ± 23.4 (1400)[b] |
| cultured on[d] | | |
| *u*-Fn | 11 ± 4 (17) | 2.8 ± 0.7 (23) |
| *s*-Fn | 13 ± 6 (20) | 3.5 ± 0.7 (25) |
| 8 M urea-treated *s*-Fn | 15 ± 4 (24) | 2.4 ± 0.6 (19) |
| *s*-Fn + heparin | 20 ± 9 (32) | 3.5 ± 1.0 (28) |
| *s*-Fn + gelatin | 13 ± 8 (21) | 2.4 ± 0.6 (19) |

[a] Fn was denatured as described under Materials and Methods. [b] Percent of control in parentheses. [c] A 50 μg/mL sample of 24K Fib 1 fragment was added to the induction medium. [d] ST-13 cells were cultured with the induction medium on culture dishes coated with 50 μg/mL *u*- or *s*-Fn, *s*-Fn treated with 8 M urea, or a mixture of *s*-Fn and heparin (25 μg/mL) or gelatin (50 μg/mL).

Table 2: Evaluation of the Fib 2 Domain Activity of *s*-Fn and MMP Digests of *s*-Fn[a]

| attractant | migrated cells/10HPF[b] | attractant | migrated cells/10HPF[b] |
|---|---|---|---|
| control (BSA) | 12 ± 4.0 | BSA | 12 ± 3.3 |
| *u*-Fn | 17 ± 6.1 | *u*-Fn | 20 ± 5.0 |
| 21K Fib 2 fragment | 50 ± 9.2 | matrilysin digest[d] | 22 ± 4.8 |
| *s*-Fn | 19 ± 4.2 | stromelysin digest[d] | 35 ± 6.5 |
| | | collagenase digest[d] | 39 ± 6.3 |
| 8 M urea-treated *s*-Fn | 14 ± 7.3 | gelatinase digest[d] | 64 ± 6.6 |
| (*s*-Fn + heparin)[c] | 21 ± 10.7 | (gelatinase digest + Fn8-12)[e] | 19 ± 8.2 |
| (*s*-Fn + gelatin)[c] | 24 + 9.8 | 21K Fib 2 fragment | 54 ± 10.3 |
| | | 70K Fib 2 fragment | 33 ± 5.6 |

[a] The Fib 2 domain activity is represented by the chemotactic activity at 0.5 nM as described under Materials and Methods. [b] Represented as the number of migrated cells at 0.5 nM of each attractant. [c] 50 μg/mL *s*-Fn incubated with 30 μg/mL heparin or gelatin was diluted to the appropriate concentrations of *s*-Fn and then assayed. Data are represented as the migrated cell number at 0.5 nM *s*-Fn. [d] Molar concentrations of the MMP digests were calculated as intact Fn used for the digestion. [e] The gelatinase digest (180 μg/mL) was mixed with protein A−Sepharose (50 μL packed volume) that was incubated with FN8-12 (150 μG), diluted to appropriate concentrations, and then assayed.

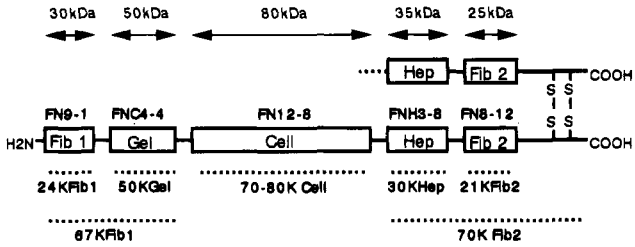


FIGURE 2: Arrangement of the functional domains as well as the alignment and apparent molecular masses of the Fn fragments used in this study. The recognition domains of the mAbs are also presented.

value ($2.75 \times 10^7$ cm$^2$/s). Urea irreversibly decreased the diffusion coefficient value of *s*-Fn ($1.728 \times 10^7$ cm$^2$/s). The Stokes radii of *u*- and *s*-Fns and the urea-treated *s*-Fn were calculated to be 14.0, 9.1, and 14.2 nm, respectively.

To determine the arrangement of the Cell, Fib 1, and Fib 2 domains in the Fn structure, we measured the immunoreactivity of the mAbs that recognized them (see Figure 2), in *u*- and *s*-Fns (Figure 3). FN12-8 moderately immunoreacted with *u*-Fn, but only weakly with *s*-Fn. The urea-treated *s*-Fn was recognized by FN12-8, and its titration curve was similar to that of *u*-Fn. When the other type of enzyme immunoassay was performed in wells coated with *s*- or *u*-Fns, the immunoreactivities of FN12-8 with *s*- and *u*-Fns were indistinguishable (data not shown), supporting the data of the surface activation of *s*-Fn.

On the other hand, either FN9-1 or FN8-12 hardly reacted with *s*-Fn and *u*-Fn, although these mAbs definitely immunoreacted with the 24K Fib 1 and 21K Fib 2 fragments (Figure 3B,C). Treating the Fns with 8 M urea or 6 M guanidine did not elicit reactivity with these mAbs, suggesting that epitopes for FN9-1 and FN8-12 are buried reversibly in the Fn structure after removal of the denaturant.

Thus, whether the cryptic activities of Fib 1, Cell, and Fib 2 domains are disclosed or not seems to depend on whether or not these domains are exposed irreversibly from the confined environments of Fn structure.

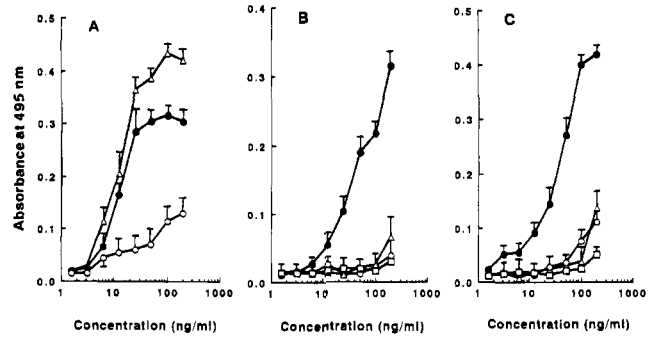


FIGURE 3: Immunoreactivity of *u*- and *s*-Fns with the domain-specific mAbs. The sandwich-type enzyme immunoassay using FN12-8 (A), FN9-1 (B), or FN8-12 (C) was constructed as described under Materials and Methods. (A) *u*-Fn (△), *s*-Fn (○), and *s*-Fn treated with 4 M urea (●). (B) 24K Fib 1 fragment (●), *u*-Fn (△), *s*-Fn (○), and *s*-Fn treated with 8 M urea (□). (C) 21K Fib 2 fragment (●), *u*-Fn (△), *s*-Fn (○), and *s*-Fn treated with 8 M urea (□). Data represent the means ± SE of four determinants.

*Activation of Quiescent Fn by Matrix Metalloproteinases (MMPs).* Activation of the Fib 1, Cell, and Fib 2 domain activities from *s*-Fn by MMPs was investigated. Figure 4 shows immunoblots of the Fn fragments in MMP digests using the domain-specific mAbs. Matrilysin did not degrade *s*-Fn into smaller fragments, and the resulting fragments were stained immunochemically by any of FN8-12, FN12-8, and FN9-1, indicating that the MMP cleaves near the carboxyl-terminal disulfide bridges to generate half a molecule of Fn. Stromelysin, gelatinase A, and collagenase degraded *s*-Fn to generate Fib 1, Cell, and Fib 2 domain-related fragments. With respect to release of the Fib 1 domain (Figure 4A), these MMPs yielded fragments of similar sizes. Among them, 50−80-kDa fragments were also immunostained with FNC4-4 (data not shown), indicating that in these fragments the Fib 1 and Gel domains remained linked. On the other hand, all the MMPs except matrilysin released Fib 2 domain-related fragments with various molecular sizes (Figure 4B). Immunoblotting using FNH3-8 (data not shown) showed that 40−70-kDa fragments were composed of Hep−Fib 2 coupled fragments. Fragments composed only of the Fib 2 domain (17−30-kDa) seemed to be generated most abundantly by gelatinase A. Immunoblots using FN12-8 showed that gelatinase A also released the Cell domain (Figure 4C).

We then quantified the Cell, Fib 1, and Fib 2 domain activities in these MMP digests. The Cell domain activity was judged by chemotactic activity (Figure 5). The matrilysin digest showed a bell-shaped chemotactic dose−response curve similar to that of *u*-Fn. In contrast, the gelatinase digest was chemotactically much more active throughout a wide range of concentrations, suggesting that several chemotactic fragments with different optimum concentrations were released. The stromelysin and collagenase digests showed similar dose−response curves (data not shown). To evaluate the contribution of Cell domain-related fragments, 70−80-kDa Cell fragments were separated from the gelatinase digest (Figure 4C), and then their chemotactic activity was examined. Figure 5B shows that the Cell fragments had much more activity than *u*-Fn, which was competed by the GRGDSP peptide (Figure 5B). Thus, *s*-FN showed Cell domain activity after urea treatment, but acquired a higher level of the activity by proteolytic fragmentation.

The Fib 1 domain activity was evaluated by the ability to stimulate adipocyte differentiation (Figure 6). No stimulatory

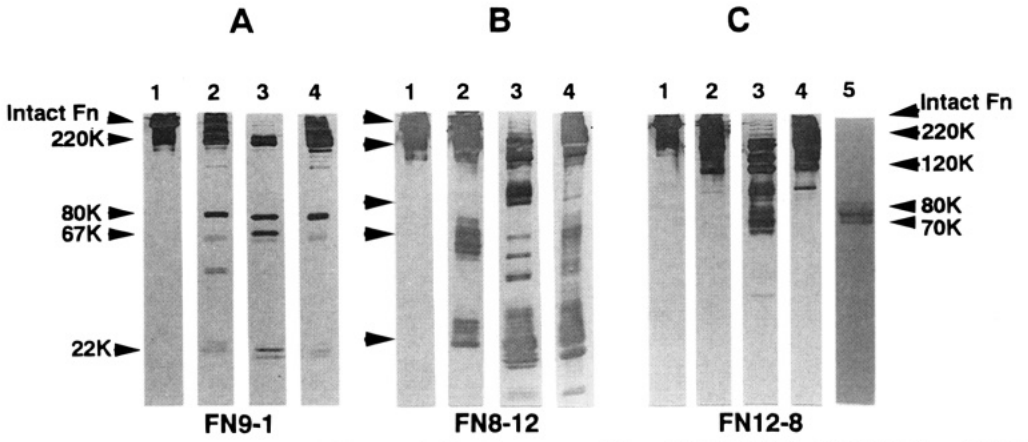


FIGURE 4: Immunoblots of Fn fragments in MMP digests of s-Fn. Immunostaining with FN9-1 (A), FN8-12 (B), and FN12-8 (C). Lanes: 1, matrilysin digest; 2, stromelysin digest; 3, gelatinase digest; 4, collagenase digest; 5, fragments separated from the gelatinase digest of *s*-Fn by passage through affinity columns of heparin− and gelatin−Sepharose.

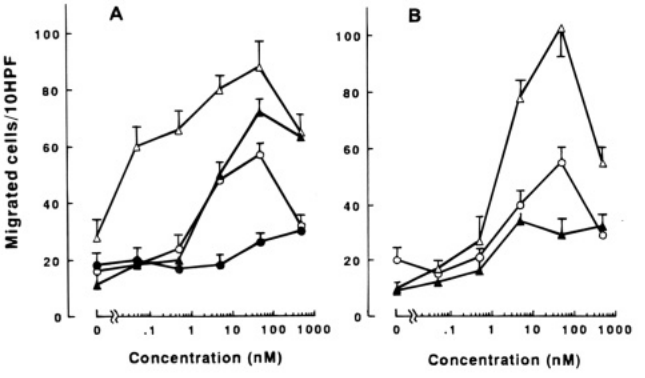


FIGURE 5: Chemotactic dose−response curves of NIH-L13 fibroblasts toward *s*-FN and its digest with MMPs. (A) *s*-Fn (●), matrilysin digest (○), and gelatinase digest (△) of *s*-Fn. The gelatinase digest (180 μg protein/mL) was incubated with protein A−Sepharose (50 μL packed volume) treated with FN8-12 (150 μg of IgG) (▲). (B) *u*-Fn (○), the Cell fragments separated from the gelatinase digest of *s*-Fn (see the legend of Figure 4) (△), and the Cell fragments mixed with GRGDSP peptide (0.2 mM) (▲). Data represent the means ± SE of three determinants. Molar concentrations of attractants were calculated assuming molecular masses of the MMP digests and Cell fragments as 420 and 80 kDa, respectively.

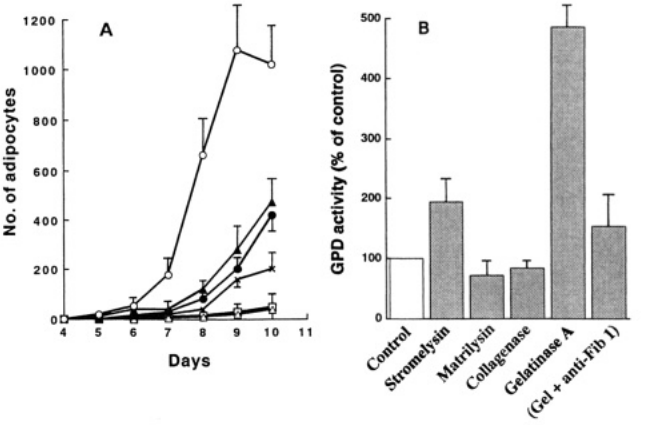


FIGURE 6: Effects of the MMP digests on adipocyte differentiation of ST-13 cells. (A) Number of adipocytes counted daily among ST-13 cells cultured with the induction medium in the absence (×) or presence of 100 μg of protein/mL of the matrilysin (△), stromelysin (●), collagenase (□), or gelatinase (○) digest of *s*-Fn and the gelatinase digest incubated with anti-Fib 1 IgG (80 μg) (▲). (B) Glycerophosphate dehydrogenase activity of ST-13 cells cultured for 10 days as described above. Data represent the means ± SE of four determinants.

effect on differentiation was observed with the matrilysin digest. On the other hand, although the MMPs except matrilysin generated similar Fib 1-related fragments (Figure 4A), only the gelatinase digest preferentially stimulated the differentiation. Since about 70% of the stimulatory activity was neutralized by immunoprecipitation with anti-Fib 1 polyclonal antibody (Figure 6), the differentiation stimulatory activity of the gelatinase digest was attributed to Fib 1-related fragments. We estimated which form was active between the fragments composed of the Fib 1 domain alone and of the Fib 1 domain bound to other domains, by comparing the Fib 1 domain activity of the 24K Fib 1 (see Figure 2) and 67K Fib 1 fragments (see Figure 2). As summarized in Table 3, the differentiation stimulatory activity of the 67K Fib 1 fragment was one-fourth and half of the 24K Fib 1 fragment as evaluated by the number of adipocytes and the glycerophosphate dehydrogenase activity, respectively. In fact, the amount of fragments composed only of the Fib 1 domain seemed to be more prominent in the gelatinase digest than in the other digests (Figure 4A). Additionally, since the MMP digests other than that of gelatinase still contained intact FN (Figure 4) that inhibited differentiation, this may

Table 3: Comparison of the Fib 1 Domain Activity between the Fragments Composed of Only Fib 1 Domain and Fib 1 and Gel Domains

| | no of adipocytes (% of control) | GPD activity[a] (% of control) |
|---|---|---|
| control | 89 ± 18 (100) | 9.5 ± 0.8 (100) |
| +24K Fib 1 fragment (2 μM) | 832 ± 136 (935) | 99 ± 14.7 (1004) |
| +67K Fib 1 fragment (2 μM) | 208 ± 40 (234) | 47 ± 10.6 (494) |
| +(24K Fib 1 + 50K Gel)[b] | 870 ± 185 (978) | 83 ± 15.0 (874) |

[a] GPD activity represented as the oxidation rate of NADH (micromoles per minute per milligram of protein). [b] Mixture of 2 μM each of the 24K Fib 1 and 50K Gel fragments.

also emphasize the differentiation stimulatory activity of the gelatinase digest. These data indicated that the Fib 1 domain activity is inhibited by adding the Gel domain. However, no significant decrease in the differentiation stimulatory activity of the 24K Fib 1 fragment was caused by the 50K Gel fragment (Table 3).

The Fib 2 domain activity of the MMP digests was assessed by measuring chemotactic activity at 0.5 nM (Table 2). The gelatinase digest also showed the highest Fib 2 domain activity which was precipitated by FN8-12 (Table 2

and Figure 5A). The 21K Fib 2 fragment was much more active in Fib 2 domain activity than the 70K Fib 2 fragment (see Figure 2). This might explain the prominence of the activity in the gelatinase digest. The 30K Hep fragment did not affect the activity of the 21K Fib 2 fragment (data not shown).

## DISCUSSION

In this study, we purified Fn without exposure to denaturants such as urea. The *s*-Fn had at least no Fib 1, Cell, and Fib 2 domain activities, whereas only the Cell domain activity was easily disclosed through a conformational change induced by denaturation or surface adsorption. Several studies have shown that the Cell domain is partially buried in the Fn structure and that it is disclosed by a conformational change [see the review by Hynes, (1986b)]. The active form of Fn is generally considered to be its insoluble ECM form. The cryptic Cell domain activity would be disclosed through incorporation of plasma Fn into ECM *in vivo*.

In contrast, any attempts to disclose the Fib 1 and Fib 2 domain activities by a conformational change have resulted in failure. The functional domains other than the Cell domain are also buried in the Fn structure. Information of the Fn structure obtained by hydrodynamic methods has indicated that internal association between domains of a different net charge may lead to back-folding of the Fn chains and to a more compact overall shape (Hormann, 1982; Williams et al., 1982). Erichson and Carrell (1983) have shown that the amino-terminal region of the Fn molecule binds to the Hep domain with very high affinity ($K_d = \sim 10^{-9}$ M). Narasimhan and Lai (1989) have shown that a sulfhydryl group situated in a type III unit which is part of the Fib 2 domain still remains buried, although another sulfhydryl group present in the Cell domain becomes exposed through adsorption to plastic beads. The data here showed that the Fib 1 and Fib 2 domains are exposed in the denaturing buffer, whereas they are folded back reversibly after removing the denaturant. Interdomain interactions may make the active regions in these domains much less accessible.

The Fn conformation has been closely linked to the binding of the functional domains with their ligands. Williams et al. (1982) have observed that the diffusion constant of Fn falls when an Fn-binding fragment of type I collagen [α1-(I)CB7] (Kleiman et al., 1978) is added, suggesting that binding of the collagen fragment to Fn leads to some unfolding of the molecule. Another investigator has analyzed conformational changes in the two Fib 1 domains on the binding of gelatin and heparin and has shown that binding of either ligand increases the affinity for the other, which is also consistent with the unfolding or binding of ligand (Homandberg, 1987). Ligand-induced conformational changes of Fn therefore should liberate the Fib 1 and Fib 2 domain activities. Since heparin stimulates the differentiation of ST-13 cells (unpublished data), we examined its effects by culturing the cells on dishes coated with a mixed solution of *s*-Fn and heparin. However, there was no changing the differentiation inhibitory activity of the Fn. That is, heparin could not liberate the Fib 1 domain activity. The Fib 1 domain activity was also not observed by incubating *s*-Fn with gelatin. On the other hand, since gelatin at a high concentration (>100 μg/mL) significantly stimulates NIH-L13 cell migration (unpublished data), the Fib 2 domain activity was assayed at a concentration (30 μg/mL) that would be low enough not to permit cell migration. The Fib 2 domain activity was not liberated by gelatin and heparin. As described previously (Fukai et al., 1993a), the Fib 1 and Fib 2 domain activities cannot be observed when the 24K Fib 1 and 21K Fib 2 fragments are coated on the substrate surfaces. Fn incorporated into the extracellular matrix as an insoluble component may be incapable of expressing the Fib 1 and Fib 2 domain activities.

We investigated the action of MMPs on the release of Fib 1 and Fib 2 domains. Among the MMPs tested, gelatinase A most effectively degraded *s*-Fn to free the Fib 1 and Fib 2 domains from linkage with adjacent domains. Prominent Fib 1 and Fib 2 domain activities of the gelatinase digest would be explained by the fact that fragments (24K Fib 1 and 21K Fib 2) composed only of the responsible domains had much higher activities than those (67K Fib 1 and 70K Fib 2) linked to their adjacent domains. This could be due to the expression of cryptic activities within the responsible domains or due to the removal of inhibitory activity in the Gel and Hep domains. The latter possibility was ruled out by adding Gel and Hep fragments. Fn bound with high affinity to ECMs surrounding ST-13 and NIH-L13 cells via the Gel and Hep domains. We speculate that the Fib 1 and Fib 2 domains in Fn are restricted in interaction with their putative cell-surface receptors by linkage with Gel and Hep domains. Regardless, determination of the active sites in Fib 1 and Fib 2 domains and their arrangement in Fn structure would provide an definitive answer. Further studies are necessary.

Since MMPs preferentially attack ECM components such as collagen, laminin, and Fn, many biological processes regulated by ECMs are affected by MMP action (Liotta et al., 1986). MMPs are presumed to play an essential role in tumor metastasis (Liotta et al., 1991). By disrupting ECM components, MMPs enable tumor cells to detach from the primary tumor, to migrate throughout connective tissue, and to infiltrate blood vessels. This study shows that MMPs, including gelatinase A, released active Fib 2 and Cell fragments, the former of which stimulates cell migration at low concentrations and the latter of which exerts a potent chemotactic activity at higher concentrations. These chemoattractive Fn fragments also stimulate the migration of malignant fibrosarcoma cell lines such as HT1080 and WI-38 VA13 (data not shown). Some of the MMP action on metastasis might be accounted for by release of the chemoattractive Fn fragments. Homandberg et al. (1992) have reported that Fn fragments containing the Fib 1 domain enhance gelatinolytic and collagenolytic proteinase release from bovine articular cartilage slices in culture. Thus, release of the chemoattractive Fn fragments triggered by MMPs could be further amplified by liberating Fib 1 domain activity. Similarly, the active Fn fragments might also be involved in other MMP effects such as embryonic development, inflammation, and tissue remodeling. Further study will be directed toward investigating whether or not active Fn fragments can be found in loci where MMPs are expressed.

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