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Recombinant Expression and Characterization of a Novel Fibronectin Isoform Expressed in Cartilaginous Tissues*[S]

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A novel fibronectin (FN) isoform lacking the segment from IIICS (type III connecting segment) through the I-10 module is expressed predominantly in normal cartilaginous tissues. We expressed and purified recombinant cartilage-type FN using a mammalian expression system and characterized its molecular and biological properties. Although FNs have been shown to be secreted as disulfide-bonded dimers, cartilage-type FN was secreted mainly as a monomer. It was less potent than plasma-type FN in promoting cell adhesion and binding to integrin $\alpha_5\beta_1$, although it was more active than plasma-type FN in binding to chondroitin sulfate E. When added exogenously, cartilage-type FN was poorly assembled into the fibrillar FN matrix, mostly because of its monomeric structure. Given that cartilage is characterized by its non-fibrillar matrix with abundant chondroitin sulfate-containing proteoglycans, it is likely that cartilage-type FN has evolved to adapt itself to the non-fibrillar structure of the cartilage matrix through acquisition of a novel mechanism of alternative pre-mRNA splicing.

Fibronectin (FN)[1] is a multifunctional glycoprotein present in the extracellular matrix as an insoluble matrix component and in circulating plasma as a soluble protein. FN plays important roles in many physiological events through its binding to cell-surface receptors such as integrins and membrane-bound heparan sulfate proteoglycans (*e.g.* syndecans) (1). FN consists of three types of homologous repeating units (types I–III) and exists as a dimer of 220–250-kDa subunits linked together by a pair of disulfide bonds located at the C-terminal end. The dimeric structure of FN is essential for its self-assembly into the extracellular matrix (2). Many functional domains, including the N-terminal heparin-1/fibrin-1 domain and the central cell-binding domain (CCBD) containing the integrin-binding Arg-Gly-Asp motif, have been shown to be involved in FN matrix assembly (2–5). Furthermore, the binding affinity of FN for integrins, particularly $\alpha_5\beta_1$, modulates the deposition of FN in the extracellular matrix (6, 7).

FN is encoded by a single gene, but exhibits molecular heterogeneity arising from alternative splicing of the primary transcript at three distinct regions termed EDA (or EIIIA), EDB (or EIIIB), and IIICS (or V) (8, 9). These alternatively spliced regions have been shown to have their own biological activities or to modulate the functions of neighboring domains (10–15). Thus, the IIICS and EDA regions bind to integrins $\alpha_4\beta_1$ (11, 12) and $\alpha_9\beta_1$ (16), thereby mediating cell adhesion to substrates, whereas insertion of EDA adjacent to the CCBD potentiates the cell-adhesive and integrin-binding activities of the CCBD possibly through altering the global conformation of FN (13). The biological function of EDB still remains elusive, but embryonic fibroblasts deficient in production of EDB-containing FNs exhibit reduced cell proliferation and FN matrix assembly *in vitro* (15). Besides these alternatively spliced regions, the III-15 and I-10 modules have been shown to be eliminated from the FN molecule along with the IIICS region by alternative RNA splicing in cartilaginous tissues (17). This novel FN isoform lacking the region encompassing IIICS through the I-10 module (designated the "V+C" region) appears to be unique to normal cartilaginous tissues because it was barely detectable in osteochondrogenic tumors (18). The function of this cartilage-specific FN isoform remains to be elucidated.

In this study, we constructed a mammalian expression plasmid for cartilage-type FN and purified the recombinant FN secreted into the conditioned medium. Comparative analyses of recombinant cartilage-type and plasma-type FNs showed that cartilage-type FN was secreted mainly as a monomer and was barely assembled into FN fibrils. It was also less potent than plasma-type FN in binding to integrin $\alpha_5\beta_1$ and in mediating cell spreading, although it exhibited a strong binding activity for chondroitin sulfate E.

EXPERIMENTAL PROCEDURES

cDNA Construction—The cDNA expression vector pAIFNC, which encodes recombinant plasma-type FN (designated rFN/C), was described previously (13). For construction of an expression vector encoding cartilage-type FN, we first isolated total RNA from human rib cartilage and amplified the FN cDNA fragment encoding III-12 through I-12 by reverse transcription-PCR. An internal BglII/NdeI fragment

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[S] The on-line version of this article (available at http://www.jbc.org) contains Supplemental Fig. 1.

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[1] The abbreviations used are: FN, fibronectin; CCBD, central cell-binding domain; rFN/C, recombinant plasma-type fibronectin; rFN/O, recombinant cartilage-type fibronectin; rFN/Cm, monomeric recombinant plasma-type fibronectin with substitution of Ser for two Cys residues in the C-terminal dimer-forming segment; CHO, Chinese hamster ovary; FBS, fetal bovine serum; mAb, monoclonal antibody; PBS, phosphate-buffered saline; GAG, glycosaminoglycan; PE-GAG, phosphatidylethanolamine-conjugated glycosaminoglycan; CS, chondroitin sulfate; IIICS, type III connecting segment.

encoding III-14 through I-12 but lacking IIICS through I-10 was excised from the amplified cDNA and substituted for the corresponding fragment in pAIFNC, yielding an expression plasmid encoding recombinant cartilage-type FN (designated rFN/O). For construction of an expression vector encoding monomeric recombinant plasma-type FN (designated rFN/Cm), pAI70F2mono, which encodes the C-terminal 37-kDa region with substitution of Ser for both Cys^{2427} and Cys^{2431} within the dimer-forming segment (19), was digested with ApaI/NspV, and the resulting 1017-bp fragment was ligated to ApaI/NspV-cleaved pBluescript containing the BamHI-cleaved fragment of pAIFNC, followed by excision of a 3316-bp BamHI fragment and insertion into BamHI-cleaved pAIFNC, yielding the expression vector pAIFNCm, which encodes rFN/Cm.

Cell Culture, DNA Transfection, and Selection of Stable Transformed Cells—Chinese hamster ovary (CHO) DG44 cells deficient in dihydrofolate reductase activity were obtained from Dr. Lawrence Chasin (Columbia University) and grown in α-minimal essential medium containing ribonucleosides/deoxyribonucleosides and 10% fetal bovine serum (FBS). The expression vectors for rFN/O, rFN/C, and rFN/Cm were cotransfected into CHO DG44 cells together with pGEMSVdhfr, which encodes a dihydrofolate reductase minigene (provided by Dr. Hiroshi Teraoka, Shionogi Research Laboratory, Shionogi and Co. Ltd., Osaka, Japan), by the calcium phosphate precipitation method (20). Selection of stable transfectants and subsequent amplification of the introduced cDNA were carried out as described (21). Stable transfectants overexpressing recombinant human FNs were maintained in α-minimal essential medium without ribonucleosides/deoxyribonucleosides in the presence of 10% FBS. Mouse embryonic fibroblasts were grown in Dulbecco's modified Eagle's medium containing 10% FBS. HT1080 human fibrosarcoma cells were obtained from the Japanese Cancer Research Resources Bank and maintained in Dulbecco's modified Eagle's medium containing 10% FBS.

Antibodies and Peptides—Monoclonal antibodies (mAbs) against human FN (136H, 119A, and 17E) and hamster FN (YFN3 and 3C12) were established in our laboratory by fusion of SP2 myeloma cells with the spleen cells of BALB/c mice immunized with human plasma FN or hamster plasma FN, respectively. The epitopes for mAbs 136H and 119A have been mapped to the III-1 module, and that for mAb 17E to the collagen/gelatin-binding domain. mAb FN1-1, which recognizes the extreme C-terminal segment containing the dimer-forming cysteines, was purchased from Takara Bio Inc. (Otsu, Japan). Function-blocking mAbs against integrin α_5 (8F1) and β_1 (4G2) subunits were established in our laboratory (13). The mAb against human integrin $\alpha_v\beta_3$ (LM609) was purchased from Chemicon International, Inc. (Temecula, CA). A polyclonal antibody against human FN was raised in rabbits by repeated immunization with purified human plasma FN emulsified in complete Freund's adjuvant. Dialyzed control mouse IgG was from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-mouse IgG and horseradish peroxidase-conjugated goat anti-rabbit IgG were from Cappel Worthington Biochemicals (Malvern, PA). The synthetic peptides GRGDSP and GRGESP were obtained from Iwaki Glass (Chiba, Japan).

Purification of Recombinant FNs and Immunoblot Analysis—CHO transfectants overexpressing recombinant FNs were maintained in α-minimal essential medium containing 1% FN-depleted FBS. The conditioned medium (~2 liters) was clarified by centrifugation, concentrated using the Labscale™ TFF system (Millipore Corp., Bedford, MA), and passed over an immunoaffinity column containing mAb 3C12 to remove endogenous hamster FN secreted by the CHO cells. The flow-through fractions were applied to an immunoaffinity column containing mAb 119A to capture recombinant FNs. Proteins bound to the columns were eluted with 0.1 M triethylamine (pH 11.5), immediately neutralized with 2 M NaH_2PO_4 (pH 3.6), and then dialyzed against phosphate-buffered saline (PBS).

For the immunoblot analysis, purified FNs were subjected to SDS-PAGE on 6% polyacrylamide gels and then transferred to nitrocellulose membranes. The membranes were blocked with 3% skim milk and stained with monoclonal antibody against human FN or hamster FN using ECL reagents (Amersham Biosciences).

Indirect Immunofluorescence Staining—Mouse embryonic fibroblasts were plated on glass coverslips placed in a 24-well culture plate in Dulbecco's modified Eagle's medium containing 10% FN-depleted FBS and cultured for 24 h. The medium was then replaced with serum-free medium containing 50 nM recombinant human FNs and incubated for another 24 h. Cells were washed three times with PBS, fixed with 3.7% paraformaldehyde, and then double-stained with mouse anti-human FN mAb 136H and rabbit anti-FN polyclonal antibody at room temperature for 1 h. After washing with PBS, cells were incubated with Alexa Fluor™ 488-conjugated goat anti-mouse IgG and Alexa Fluor™ 546-conjugated goat anti-rabbit IgG at a dilution of 1:400 (Molecular Probes, Inc., Eugene, OR) at room temperature for 1 h. The labeled cells were washed three times with PBS, mounted in PermaFluor Aqueous Mountant (Thermo Shandon, Pittsburgh, PA), and examined with a Zeiss LSM5 PASCAL confocal laser microscope.

Cell Spreading Assay—Cell spreading assays were performed using 96-well microtiter plates (Maxisorp, Nunc, Roskilde, Denmark) coated with various concentrations of recombinant FNs as specified and blocked with 1% heat-denatured bovine serum albumin. HT1080 cells detached with 1 mM EDTA at 37 °C were plated on the plates at a density of 3×10^4 cells/ml and incubated for 30 min at 37 °C in a CO_2 incubator. For cell adhesion inhibition assays, cells were preincubated with function-blocking mAbs against different types of integrins or synthetic peptides in serum-free medium at a density of 3×10^5 cells/ml at 4 °C for 30 min before plating. Nonadherent cells were removed by washing with serum-free medium, and attached cells were fixed with 3.7% paraformaldehyde and then stained with Diff-Quick (International Reagent Corp., Kobe, Japan). Cells showing a well spread morphology (*i.e.* cells that had become flattened with their long axis more than twice the diameter of their nucleus) were counted per square millimeter.

Purification of Integrin $\alpha_5\beta_1$ and Integrin-containing Liposome Binding Assay—Integrin $\alpha_5\beta_1$ was purified and reconstituted into liposomes as described by Pytela *et al.* (22). Briefly, fresh human placental tissue was extracted with Tris-buffered saline (25 mM Tris-HCl (pH 7.5), 0.13 M NaCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$) containing 100 mM octyl glucoside and 1 mM phenylmethylsulfonyl fluoride. The extract was applied to a Sepharose affinity column conjugated with 155/145-kDa thermolysin fragments containing the CCBD. The integrin $\alpha_5\beta_1$ bound to the affinity column was eluted with Tris-buffered saline containing 1 mM GRGDSP peptide. The eluted integrin was mixed with egg yolk lecithin containing [^{3}H]dipalmitoylphosphatidylcholine (PerkinElmer Life Sciences) and then dialyzed overnight against Tris-buffered saline containing 2 mM $CaCl_2$ and 0.5 mM $MgCl_2$ at 4 °C. The reconstituted integrin-containing liposomes were added to microtiter wells coated with recombinant FNs (50 nM) and incubated for 6 h at room temperature. In some experiments, recombinant FNs were immobilized on microtiter wells via mAb 119A preadsorbed on the plates. The wells were washed three times with Tris-buffered saline, and then bound liposomes were solubilized with 1% SDS, followed by quantification of the radioactivity with a Packard Tri-Carb 1500 liquid scintillation analyzer.

Heparin Binding Assay—Heparin-binding activity was assessed by heparin affinity chromatography using HiTrap™ heparin (Amersham Biosciences AB, Uppsala, Sweden). Purified recombinant FNs were applied to a HiTrap heparin column (1-ml volume), which had been equilibrated with 10 mM phosphate buffer containing 0.14 M NaCl, and eluted with a gradient of 0.14–1.0 M NaCl (flow rate of 0.1 ml/min). The protein concentration in each fraction was monitored by the absorbance at 280 nm.

Glycosaminoglycan (GAG) Binding Assay—Phosphatidylethanolamine-conjugated glycosaminoglycans (PE-GAGs) were synthesized as described previously (23). The Maxisorp 96-well microtiter plates were coated with 20 μg/ml PE-GAG at 4 °C overnight, followed by blocking with 1% denatured bovine serum albumin for 1 h at 37 °C. After washing the wells three times with PBS, recombinant FNs (20 nM) were incubated with the PE-GAG-coated wells for 2 h at room temperature. The amounts of FN bound to the PE-GAGs were quantified by enzyme-linked immunosorbent assay using anti-human FN mAb 136H and horseradish peroxidase-conjugated anti-mouse IgG.

RESULTS

Cartilage-type FN Is Secreted as a Monomer—The structures of the recombinant human FN isoforms used in this study are illustrated in Fig. 1. These full-length FN isoforms are identical except for the alternatively spliced modules and two Cys residues within the C-terminal dimer-forming segment. A novel FN isoform lacking IIICS through the I-10 module has been shown to be expressed in normal cartilaginous tissues (17). To elucidate the physiological functions of this cartilage-type FN, we constructed an expression vector for this isoform and co-transfected CHO DG44 cells with the vector and a selection plasmid encoding a dihydrofolate reductase minigene. The resulting stable transfectants were treated with increasing concentrations of methotrexate to amplify the introduced recombinant genes. Immunoblot analyses of the conditioned medium of the transfectants showed that rFN/O was secreted into the medium (data not shown).

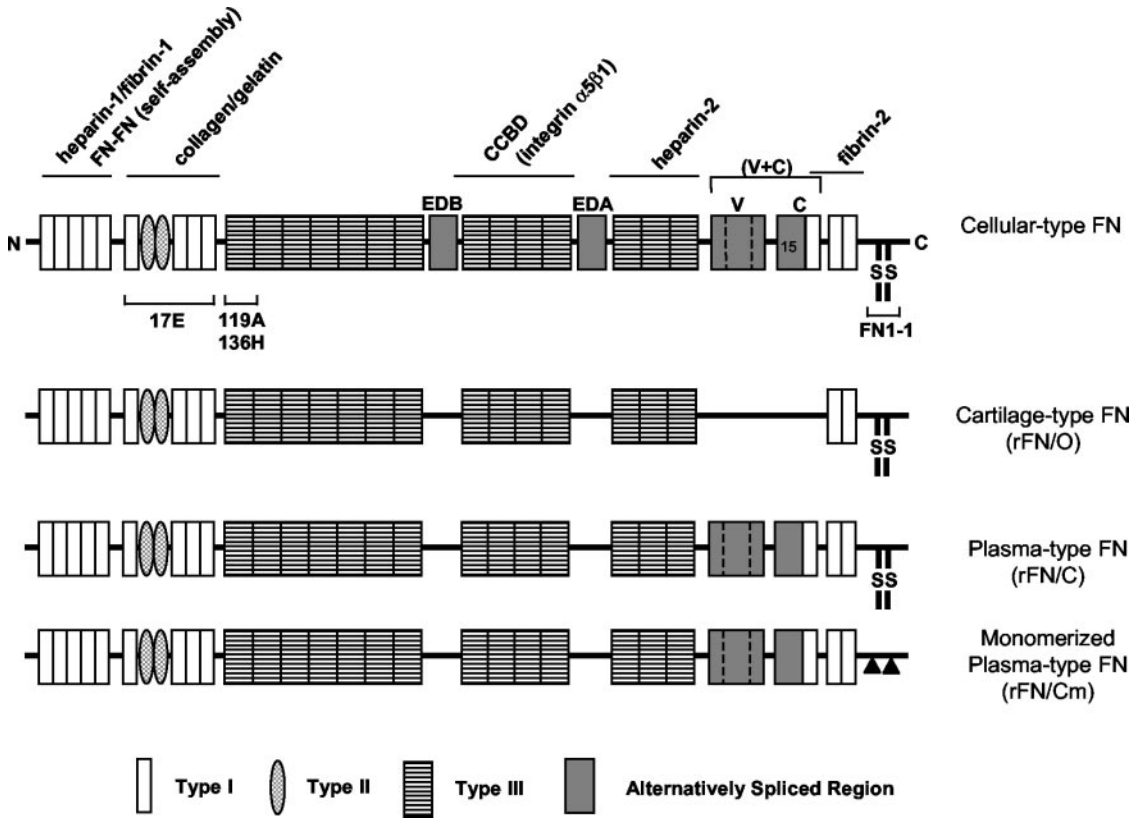

FIG. 1. **Structures of recombinant FNs.** The modular structures of the recombinant FNs are shown schematically on the basis of the internally homologous repeating units (types I–III). Alternatively spliced regions are shown as *gray boxes*. Cartilage-type FN lacks all of these regions. Two Cys-to-Ser substitutions within the C-terminal dimer-forming segment are indicated by *arrowheads*. Functional domains that interact with heparin (heparin-1 and heparin-2), fibrin (fibrin-1 and fibrin-2), collagen/gelatin, integrin $\alpha_5\beta_1$ (the CCBD), and FN itself (*FN-FN*, *self-assembly*) are indicated above the schemes. The epitope regions recognized by mAbs 119A, 136H, 17E, and FN1-1 used in this study are indicated by *brackets*.

rFN/O was purified from the conditioned medium of stable transfectants by immunoaffinity chromatography using anti-human FN mAb 119A, which recognizes the III-1 module. Purified rFN/O gave a single band with an apparent molecular mass of ~220 kDa under reducing conditions, which is significantly smaller than the molecular masses of the bands given by both rFN/C and FN purified from human plasma (Fig. 2*A*, *left panel*). Unexpectedly, rFN/O still migrated around the 200-kDa region under nonreducing conditions, whereas rFN/C and plasma FN gave bands in the ~500-kDa region, indicative of their dimeric nature (Fig. 2*A*, *right panel*). These results indicate that rFN/O was secreted predominantly as a monomer. The failure of rFN/O to form a dimer does not seem to be due to the removal of the C-terminal dimer-forming segment by proteolytic cleavage because rFN/O retained the reactivity with mAb FN1-1, which recognizes the extreme C-terminal segment (Fig. 2*B*, *right panel*). The absence of CHO cell-derived hamster FN in the purified recombinant FNs was also confirmed by immunoblot analysis with hamster FN-specific mAb YFN3 (Fig. 2*B*, *middle panel*).

Decreased Cell-adhesive Activity of Cartilage-type FN—The structure of cartilage-type FN raises the question of whether cartilage-type FN and other FN isoforms are functionally different. The monomeric configuration and the absence of the III-15 and I-10 modules might individually influence the biological functions of FNs. To examine the effect of the monomeric configuration, we produced a monomeric form of rFN/C by replacing two Cys residues in the dimer-forming segment with Ser (see Fig. 1). As expected, the resulting monomeric rFN/C (rFN/Cm) was secreted as a monomer (Fig. 3*A*). To explore the physiological functions of cartilage-type FN, we compared the cell-adhesive activity of rFN/O with those of rFN/C and rFN/Cm in cell spreading assays using HT1080 cells. When HT1080 cells were plated on substrates coated with rFN/O, the cells attached and spread on the substrates, although rFN/O was slightly less potent than rFN/C and rFN/Cm in promoting cell spreading (Fig. 3*B*). The concentration of FN in the coating solution required to attain the maximal level of cell spreading was ~10 nM for rFN/C and rFN/Cm, but ~20 nM for rFN/O. The reduced cell-spreading activity of rFN/O is unlikely to be due to its monomeric nature because rFN/Cm was as equally active as dimeric rFN/C in cell spreading assays despite its monomeric configuration.

When recombinant FNs are directly immobilized on plastic plates, the native conformation in solution might be transformed into an elongated conformation through hydrophobic interaction with the polystyrene substrates (24, 25). To avoid such conformational change imposed by direct coating onto plastic substrates, FNs were indirectly immobilized via anti-FN mAb 119A, which recognizes the III-1 module, for comparison of their cell-spreading activities. The difference in cell-spreading activity between rFN/O and rFN/C was more pronounced when FNs were indirectly immobilized on the substrates (Fig. 3*C*). Similar results were obtained when recombinant FNs were captured with another anti-FN mAb recognizing the collagen/gelatin-binding domain (data not shown).

Cartilage-type FN Binds to Integrin $\alpha_5\beta_1$ via the RGD Motif—The RGD motif in the CCBD is retained in cartilage-type FN (Fig. 1), making it likely that integrin $\alpha_5\beta_1$ is the major receptor mediating cell adhesion to cartilage-type FN, as is the case with other FN isoforms. Indeed, adhesion of HT1080 cells to rFN/O was strongly inhibited by preincubation of the cells

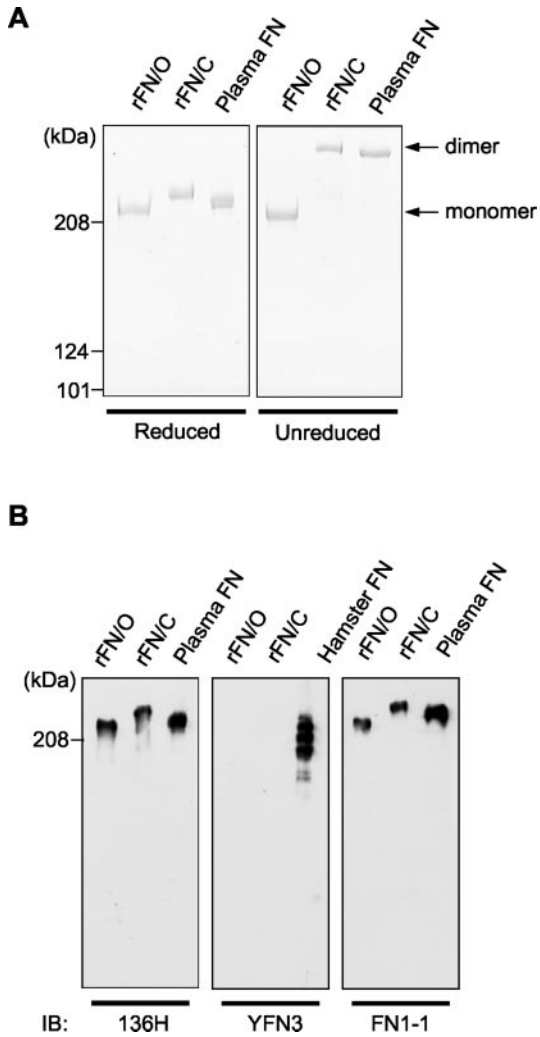

FIG. 2. **SDS-PAGE and immunoblot analysis of recombinant FNs.** *A*, purified recombinant FNs (rFN/O and rFN/C) and human plasma FN were subjected to SDS-PAGE under reducing (*left panel*) or nonreducing (*right panel*) conditions and visualized by Coomassie Blue staining. Protein (500 ng) was applied to each lane. The positions of the dimeric and monomeric forms of FNs are indicated to the right. The positions of the molecular mass markers are shown to the *left*. *B*, purified FNs (0.1 μg/lane) were subjected to SDS-PAGE under reducing conditions, followed by immunoblotting (*IB*) with the following anti-FN mAbs: 136H, which recognizes the III-1 module of human FN (*left panel*); YFN3, which recognizes hamster FN (*middle panel*); and FN1-1, which recognizes the extreme C-terminal region of human FN (*right panel*). Hamster FN was purified from hamster serum by gelatin affinity chromatography.

with function-blocking mAbs against the integrin α_5 and β_1 subunits, but not with those against integrin $\alpha_v\beta_3$ (Fig. 4*A*). Consistent with the role of integrin $\alpha_5\beta_1$ as the major receptor for rFN/O, adhesion of HT1080 cells to rFN/O was almost completely inhibited with 1 mM GRGDSP peptide, but not with 1 mM GRGESP peptide or 50 nM GRGDSP peptide (Fig. 4*B*).

Decreased Affinity of Integrin $\alpha_5\beta_1$ for Cartilage-type FN—Our data show that cartilage-type FN is less active in promoting cell spreading than plasma-type FN. Given that integrin $\alpha_5\beta_1$ is the major adhesive receptor for cartilage-type FN, we hypothesized that the decreased cell-spreading activity of rFN/O was due to its decreased affinity for integrin $\alpha_5\beta_1$. To explore this possibility, integrin $\alpha_5\beta_1$ was purified from human placenta and reconstituted into [^{3}H]phosphatidylcholine-containing liposomes to determine its binding activity for various recombinant FNs by solid-phase integrin-containing liposome binding assays. We found that integrin $\alpha_5\beta_1$-containing liposomes bound less avidly to rFN/O than to rFN/Cm or rFN/C (Fig. 5). The difference in the integrin $\alpha_5\beta_1$-containing liposome-binding activity between cartilage-type and plasma-type FNs became more pronounced when FNs were captured on the substrates via an anti-FN mAb instead of being directly coated onto the substrates (Fig. 3, *B* and *C*), consistent with the difference observed in the cell spreading assays. The binding of integrin $\alpha_5\beta_1$-containing liposomes to recombinant FNs was completely blocked by the anti-integrin α_5 mAb (data not shown), indicating that the reduced cell-spreading activity of cartilage-type FN was due primarily to the decreased binding affinity for integrin $\alpha_5\beta_1$.

Cartilage-type FN Binds Strongly to Chondroitin Sulfate E—Not only integrin $\alpha_5\beta_1$, but also heparan sulfate proteoglycans or other proteoglycans on cell surfaces have been shown to mediate interactions of cells with the FN matrix (26, 27). Thus, the reduced cell-spreading activity of cartilage-type FN may result from its decreased affinity for heparan sulfate proteoglycans. To explore the possible involvement of surface heparan sulfate proteoglycans in the reduced cell-spreading activity of cartilage-type FN, the affinity of recombinant FNs for heparin was assessed by heparin affinity chromatography using elution with a linear gradient of NaCl. The NaCl concentration needed to elute bound recombinant FNs was taken as an index to estimate their affinity for heparin. There were no significant differences in the elution profiles among rFN/O, rFN/Cm, and rFN/C, indicating that cartilage-type FN bound to heparin with an affinity comparable with that of plasma-type FN (Fig. 6).

The binding activities of recombinant FNs for other GAG chains were also determined by solid-phase binding assays using a panel of PE-GAGs (23). Lipid moieties were attached to facilitate adsorption of GAGs onto hydrophobic plastic surfaces. No significant difference was observed between plasma-type and cartilage-type FNs in the binding to phosphatidylethanolamine-conjugated heparin (Fig. 7), confirming the results obtained by heparin affinity chromatography. In contrast, none of the recombinant FNs examined showed significant binding to chondroitin sulfate (CS) A, CS-C, CS-D, dermatan sulfate, or hyaluronic acid, consistent with previous reports (28, 29). Interestingly, rFN/O exhibited significant binding to CS-E, containing 4,6-disulfated *N*-acetylgalactosamine residues, although dimeric rFN/C was only moderately active in binding to CS-E, and rFN/Cm was barely active. Differential GAG binding between rFN/O and rFN/Cm, both in the monomeric configuration, was also observed for binding to heparan sulfate; rFN/O, but not rFN/Cm, was capable of binding to heparan sulfate. rFN/C was also active in binding to heparan sulfate, possibly because of its divalent structure.

Matrix Assembly of Cartilage-type FN—Dimerization through disulfide bonds near the C terminus has been shown to be essential for FN matrix assembly (2). Because of the monomeric nature of rFN/O, we assumed that cartilage-type FN could not assemble into the FN matrix. To explore this possibility, rFN/O, rFN/Cm, and rFN/C were incubated with mouse embryonic fibroblasts for 24 h to see whether they could co-assemble with endogenous mouse FN into the FN matrix. Despite the extensive matrix assembly of mouse FN, rFN/O was only marginally incorporated into the FN matrix, whereas rFN/C was fully capable of assembling into the FN matrix (Fig. 8). rFN/Cm exhibited very weak matrix-assembling activity due to its monomeric configuration, but it was reproducibly more active than rFN/O, suggesting that the nearly complete loss of matrix-assembling activity in cartilage-type FN was not due simply to its monomeric configuration, but rather the combined effects of the monomeric configuration and reduction of either the integrin $\alpha_5\beta_1$-binding or ill-defined self-binding activity.

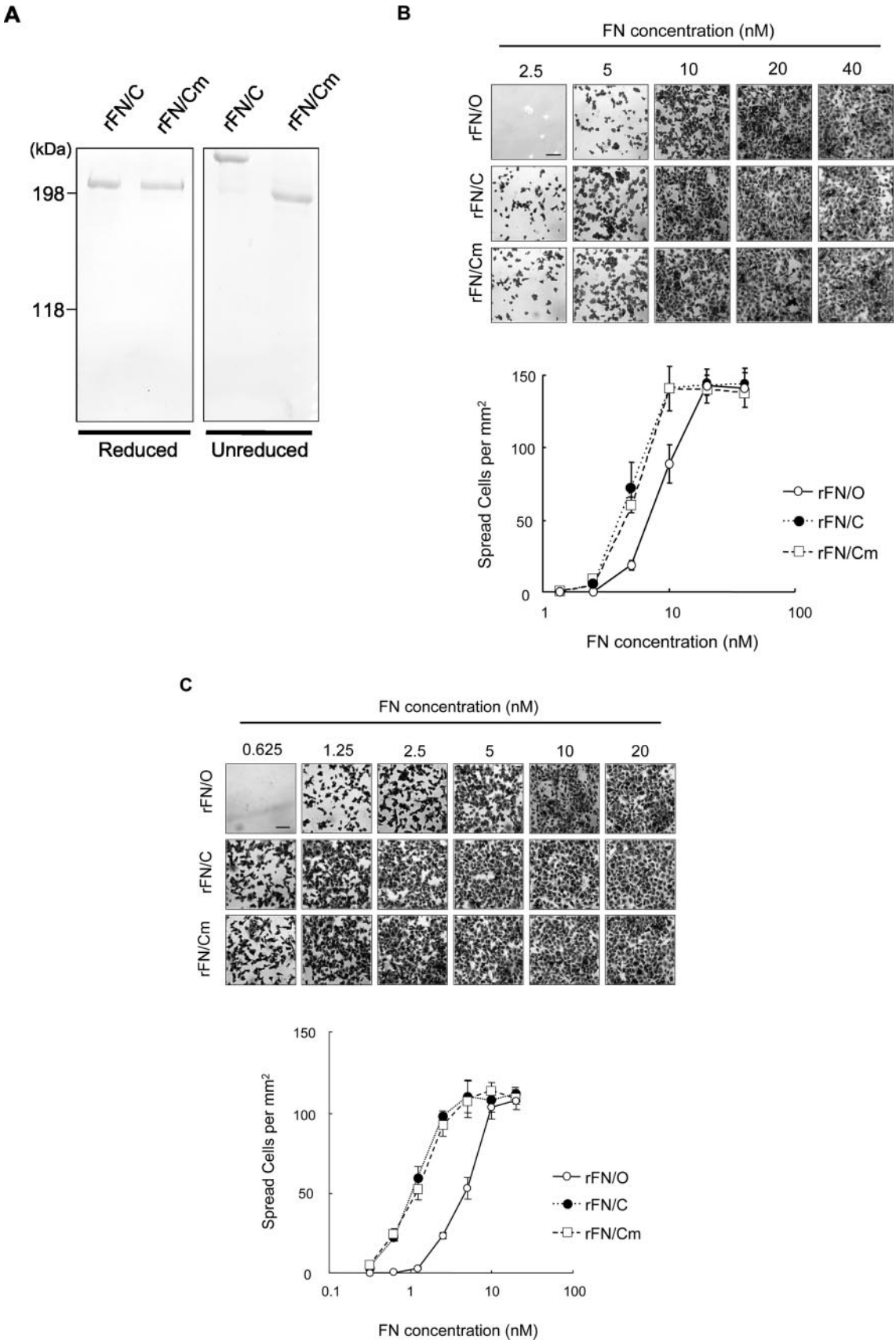

FIG. 3. **Attachment and spreading of HT1080 cells on FN-coated substrates.** *A*, rFN/Cm and wild-type rFN/C were subjected to SDS-PAGE under reducing (*left panel*) or nonreducing (*right panel*) conditions and stained with Coomassie Blue. *B*, shown is the dose dependence of the spreading of HT1080 cells on recombinant FNs directly adsorbed onto the substrates. HT1080 cells (4×10^4 cells/well) were seeded on 96-well microtiter plates coated with various concentrations of rFN/O (○), rFN/C (●), rFN/Cm (□) and incubated for 30 min at 37 °C. The attached cells were fixed with 3.7% formaldehyde and stained with Diff-Quick. *Scale bar* = 100 μm. Spread cells were quantified as described under "Experimental Procedures." *C*, shown is the dose dependence of the spreading of HT1080 cells on recombinant FNs indirectly immobilized via mAb 119A to avoid conformational change in the FN molecules due to adsorption onto the hydrophobic polystyrene substrates. Spread cells were quantified as described under "Experimental Procedures."

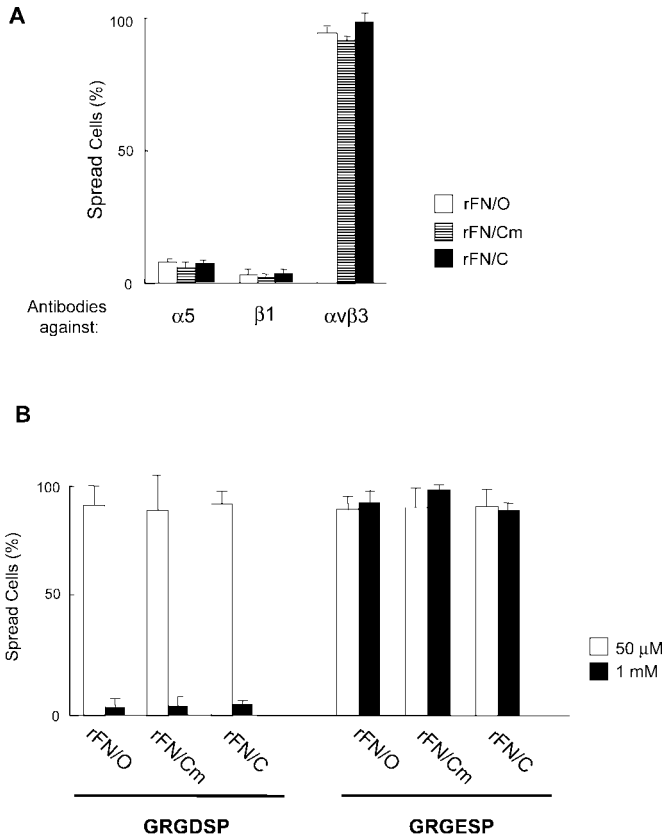

FIG. 4. **Effects of function-blocking anti-integrin mAbs and synthetic RGDS peptides on cell spreading mediated by recombinant FNs.** *A*, HT1080 cells were seeded on 96-well plates precoated with 40 nM rFN/O (*open bars*), rFN/Cm (*striped bars*), or rFN/C (*closed bars*) in the presence or absence of function-blocking mAbs (10 μg/ml) against integrins α_5 (8F1), β1 (4B2), and $\alpha_v\beta_3$ (LM609), followed by incubation at 37 °C for 30 min. The attached cells were fixed and stained, and then spread cells were counted as described under "Experimental Procedures." The number of spread cells was counted per square millimeter and is expressed as a percentage of the control cells (*i.e.* cells spread in the absence of mAbs). *Error bars* indicate standard deviation (n = 9). *B*, HT1080 cells were incubated on 96-well plates precoated with recombinant FNs as described for *A*, except that synthetic peptide GRGDSP or GRGESP (50 μM (*open bars*) or 1 mM (*closed bars*) was included instead of the anti-integrin mAbs. The number of spread cells was counted and is expressed as a percentage of the control cells (*i.e.* cells spread in the absence of peptides). *Error bars* indicate standard deviation (n = 9).

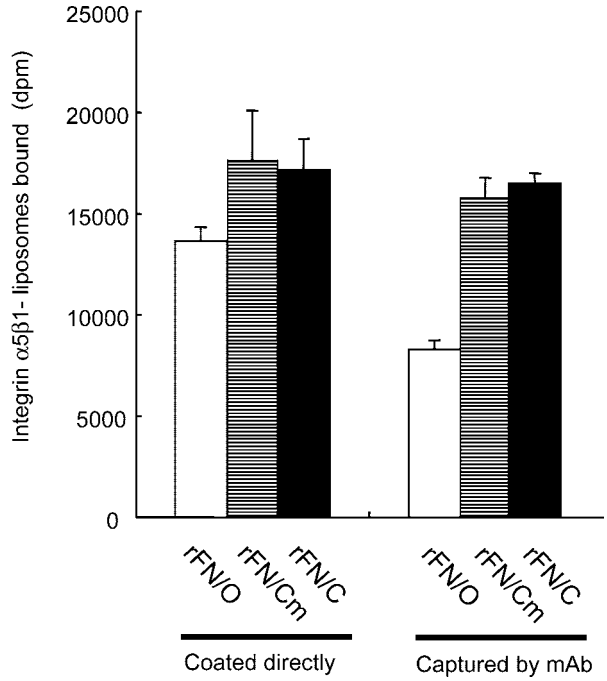

FIG. 5. **Binding of integrin $\alpha_5\beta_1$ to recombinant FNs.** Integrin $\alpha_5\beta_1$ reconstituted into [^{3}H]phosphatidylcholine-containing liposomes was incubated in 96-well plates precoated with 50 nM rFN/O (*open bars*), rFN/Cm (*striped bars*), or rFN/C (*closed bars*) for 6 h at room temperature. In some experiments, recombinant FNs were indirectly captured on the plates via mAb 119A to retain their native conformation. The quantities of bound integrin $\alpha_5\beta_1$-containing liposomes are expressed as a percentage of the total input radioactivity after subtraction of the radioactivity bound to control plates coated with bovine serum albumin only. *Error bars* indicate standard deviation (n = 5).

DISCUSSION

The first feature of cartilage-type FN is that it is secreted as a monomer. All other FN isoforms identified so far, except for some recombinant FNs lacking the IIICS region (30) or the fibrin-2 domain (31), are secreted as dimers. Our results are apparently contradictory to previous reports that rat deminectin lacking the V+C region is expressed predominantly as a homodimer (32, 33). This apparent discrepancy could be due to the N-terminal truncation in deminectins, which would likely induce a global conformational change in the FN subunits, thereby modifying their dimerizing potential. Although we have not yet examined the monomer/dimer ratio of FNs in human cartilage, Burton-Wurster *et al.* (33) reported that >80% of cartilage-type FN extracted from canine cartilage exists as a monomer, making it likely that the monomeric configuration of our rFN/O is not an artifact, but rather a reflection of a general feature of cartilage-type FNs in different species.

The second feature of cartilage-type FN is its reduced integrin-binding activity. Many lines of evidence indicate that integrin $\alpha_5\beta_1$ binds to the CCBD consisting of the III-8 through III-10 modules (13, 34). Because the CCBD remains intact after cartilage-specific alternative RNA splicing, the reduced integrin binding of cartilage-type FN could be due to either global or local conformational changes in the FN molecule, resulting in reduced accessibility and/or binding affinity of integrin $\alpha_5\beta_1$ for the CCBD. The dependence on the global conformation of the integrin-binding activity of cartilage-type FN was implicated by the differences in the integrin $\alpha_5\beta_1$-binding activities of rFN/O either directly adsorbed onto the substrates or indirectly immobilized via substrate-adsorbed antibodies. Direct adsorption of FNs onto hydrophobic polystyrene surfaces has been shown to induce a global conformational change in the molecules, converting their compact shape into a more extended conformation (24, 25). rFN/O directly adsorbed onto the substrates was more active in binding to integrin $\alpha_5\beta_1$ than rFN/O indirectly immobilized via antibodies, lending support to the possibility that the reduced integrin binding of rFN/O results from a global conformational change in the FN molecule imposed by the removal of the V+C region. Consistent with this possibility, the reactivities of a panel of mAbs against human FN were found to be different between cartilage-type and plasma-type FNs (Supplemental Fig. 1 (41)). mAbs recognizing the CCBD bound less to cartilage-type than to plasma-type FN, although the mAbs recognizing III-1 and III-13 modules bound equally to cartilage-type and plasma-type FNs, demonstrating that the accessibility of the CCBD for mAbs was reduced upon removal of the V+C region.

The third feature of cartilage-type FN is enhanced binding to CS-E. Several lines of evidence have indicated that FNs bind to CS through the C-terminal heparin-binding domain consisting of the III-12 through III-14 modules (29, 35). Because the C-terminal heparin-binding domain remains intact in cartilage-type FN, it seems likely that the enhanced CS-E binding of

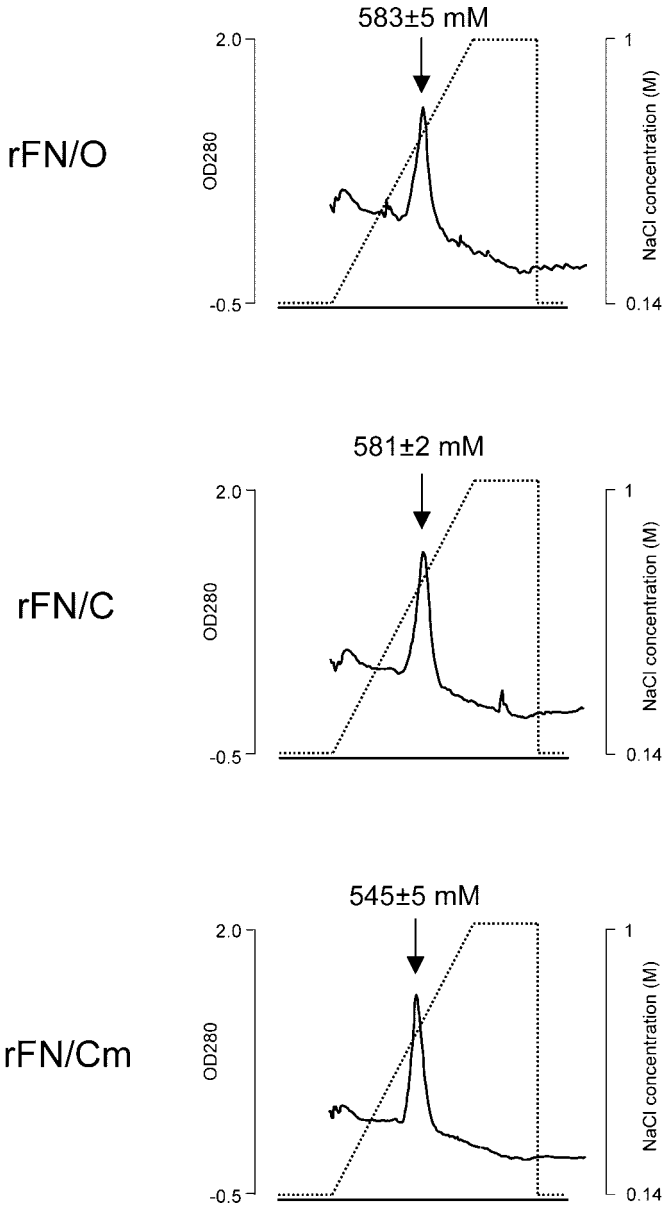

FIG. 6. **Heparin binding of recombinant FNs.** Recombinant FNs (~100 μg) were applied to a HiTrap™ heparin column, and bound FNs were eluted with a linear gradient of NaCl from 0.14 to 1 M in 10 mM phosphate buffer (pH 7.4). Eluates were monitored by the absorbance at 280 nm. The NaCl concentrations at the peak positions are indicated above each elution profile. All data represent the mean ± S.D. (n = 3).

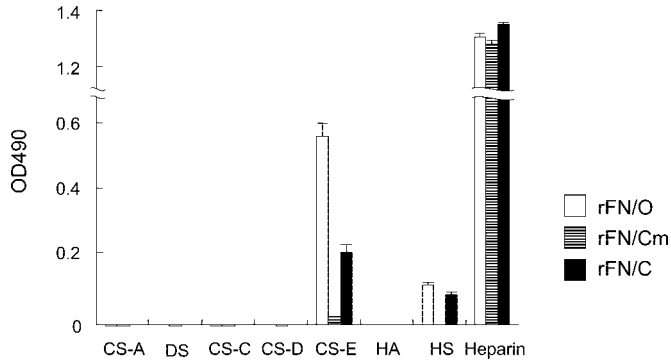

FIG. 7. **Binding of recombinant FNs to GAGs.** Microtiter plates precoated with various PE-GAGs were blocked with 2% heat-denatured bovine serum albumin and incubated with 20 nM recombinant FNs at room temperature for 2 h. Bound FNs were quantified by enzyme-linked immunosorbent assay using anti-human FN mAb 136H and horseradish peroxidase-conjugated goat anti-mouse IgG. *Error bars* indicate standard deviation (n = 5). *DS*, dermatan sulfate; *HA*, hyaluronic acid; *HS*, heparan sulfate.

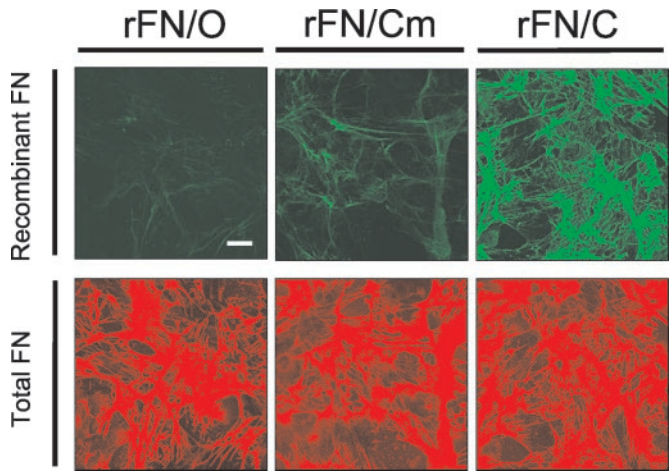

FIG. 8. **Matrix assembly of recombinant FNs.** Mouse embryonic fibroblasts were precultured to confluency on glass coverslips for 24 h and then incubated with fresh serum-free medium containing 50 nM recombinant FNs for another 24 h. Cells were fixed and stained with anti-human FN mAb 136H (*upper panels*) or rabbit anti-FN serum (1:2000 dilution; *lower panels*) as described under "Experimental Procedures." *Scale bar* = 10 μm.

cartilage-type FN is due to the conformational perturbation resulting from the removal of the V+C region, as also speculated for the reduced binding of cartilage-type FN to integrin $\alpha_5\beta_1$. Relevant to this possibility is the previous observation that deminectin exhibits higher binding affinity for CS compared with intact FN (29). Enhanced CS binding of deminectin has been ascribed to the regulatory role of the N-terminal region, which may modulate the CS-binding activity of the C-terminal heparin-binding domain through either direct domain-domain interaction or modulation of the global conformation, thereby leading to the unmasking of cryptic CS-binding sites other than the C-terminal heparin-binding domain.

The enhanced binding of cartilage-type FN to CS-E raises the possibility that cartilage-type FN forms a complex with aggrecan, the major CS-containing proteoglycan in cartilage, because more than half of the terminal *N*-acetylgalactosamine residues of aggrecan have been shown to be 4,6-disulfated, hence existing as CS-E (36). Recently, Gendelman *et al.* (37) reported that cartilage-type FN has a higher affinity for decorin compared with plasma-type FN at a low salt concentration (*i.e.* 30 mM NaCl). The binding was considered to be mediated by the CS chains because FN binding to decorin was abolished by chondroitinase ABC treatment. It remains unclear, however, whether binding of cartilage-type FN to the CS chains of decorin persists even under physiological ionic conditions.

The fourth feature of cartilage-type FN is its poor ability to assemble into the fibrillar FN matrix, which is attributable to its monomeric configuration. It has been well documented that monomeric forms of recombinant FNs possess very poor matrix-assembling activity, mostly due to their lack of self-polymerizing activity (2, 19). It should be noted, however, that rFN/O was less active than rFN/Cm in assembling into the FN matrix of fibroblasts, even though both recombinant FNs existed as monomers. The difference in their matrix-assembling activity could be due to the absence of the I-10 module in rFN/O because deletion of the I-10 module from "mini-FN," which consists of the N-terminal 70-kDa region and the C-terminal 37-kDa region, the latter of which encompasses the III-15 mod-

ule through the C terminus, results in a small, yet reproducible reduction of its FN matrix-assembling activity (19). The involvement of the fibrin-2 domain, which consists of the I-10 through I-12 modules, in FN matrix assembly was previously documented by Sottile and Mosher (31), consistent with the potential role of the I-10 module in FN matrix assembly. The reduced integrin $\alpha_5\beta_1$ binding of rFN/O may also contribute to the difference in the matrix-assembling activity because the binding of FN to the cell-surface integrin receptors, particularly $\alpha_5\beta_1$, has been considered to be the initial event in a cascade of FN matrix assembly processes (38–40).

Recently, Chen *et al.* (32) reported that rat rFN/O can assemble into a fibrillar matrix as efficiently as plasma-type and cellular-type FNs. The authors did not refer to whether the rFN/O was secreted as a dimer or monomer, making it difficult to reconcile the poor FN matrix-assembling activity of our cartilage-type FN with the apparently normal matrix assembly of their rFN/O. This discrepancy could be due to a species difference in the matrix-assembling activity between rat and human cartilage-type FNs or reflect a difference in the cells used for the assays of the FN matrix-assembling activity. Chen *et al.* used mouse embryonic fibroblasts deficient in FN expression; therefore, exogenous FNs did not compete with endogenously secreted FNs for the cell-surface FN receptors, whereas we used wild-type embryonic fibroblasts, which secrete a large amount of endogenous cell-type FNs, which did compete with exogenous FNs, thereby blocking the assembly of exogenously added cartilage-type FN.

The cartilage matrix is characterized by its amorphous, nonfibrillar structure composed of collagen II and CS-containing proteoglycans, *e.g.* aggrecan. Given the monomeric configuration of cartilage-type FN and its poor FN matrix-assembling activity, it is conceivable that cartilage-type FN assembles into the cartilage matrix through binding to other constituents, particularly aggrecan. Enhanced binding of cartilage-type FN to CS-E supports this possibility. Combined with the reduced integrin $\alpha_5\beta_1$-binding activity, the monomeric configuration of cartilage-type FN may thus contribute to the mechanical and functional properties of the cartilage matrix characterized by its resilience and cytostatic environment for the resident cells. Further studies on the physiological roles of cartilage-type FN and the structural basis of its monomeric configuration and unique biological activities should shed light on the molecular basis of the structure and functions of the cartilage matrix.

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Recombinant Expression and Characterization of a Novel Fibronectin Isoform Expressed in Cartilaginous Tissues

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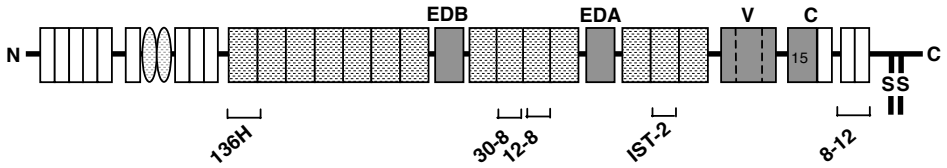
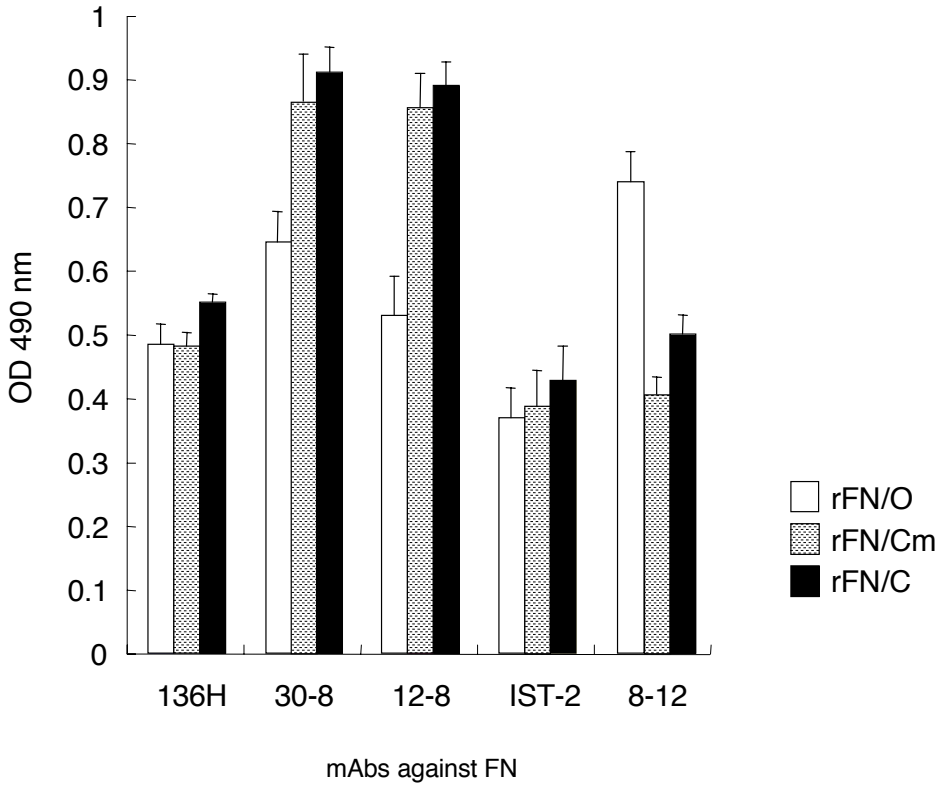
Supplemental Figure 1: Kozaki *et al*.





Supplemental Fig. 1. Probing the conformational difference between cartilage-type and plasma-type FNs using a panel of anti-human FN mAbs with defined specificities. Ninety-six-well microtiter plates were coated with gelatin (50 μg/ml) overnight at 4°C, blocked with 1% denatured BSA for 1 h, and incubated with recombinant FNs (10 nM) at room temperature for 2 h to capture them on the wells. After washing with PBS three times, the wells were incubated with a panel of anti-human FN mAbs (at 1:3,000 dilution) at room temperature for 1 h, followed by a standard immunosorbent assay using HRP-conjugated goat anti-mouse IgG. mAbs used were: 136H recognizing III-1 module; 30-8 recognizing III-9 (41); 12-8 recognizing III-10 (41); IST-2 recognizing III-13 (K.S., unpublished); and 8-12 recognizing the fibrin-2 domain (41). Although 136H and IST-2 bound equally to both cartilage-type and plasma-type FNs, the mAbs recognizing the CCBD, i.e., 30-8 and 12-8, bound less to cartilage-type FN than plasma-type FN. The reactivity of mAb 8-12 recognizing the C-terminal fibrin-2 domain was rather higher with cartilage-type than plasma-type FN. These differences in the reactivities of a panel of mAbs with different FN isoforms indicate that the conformation of the CCBD as well as the fibrin-2 domain may differ between cartilage-type and plasma-type FNs.