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 FNAs 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 α5β1, 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 nonmonomeric 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) 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 α5β1, 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 α5β1 (11, 12) and α5β1 (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 (15). The biological function of EDB still remains elusive, but embryonic fibroblasts deficient in production of EDB-containing FNAs exhibit reduced cell proliferation and FN matrix assembly in vitro (15). Besides these alternatively spliced regions, the II-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 FNAs 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 α5β1 and in mediating cell spreading, although it exhibited a strong binding activity for chondroitin sulfate E.

EXPERIMENTAL PROCEDURES

**cDNA Construction**—The cDNA expression vector pAlFNC, which encodes recombinant plasma-type FN (designated rFNc), was described previously (15). For construction of an expression vector encoding cartilage-type FN, we first isolated total RNA from human rib cartilage and amplified the FNc cDNA fragment encoding III-12 through I-12 by reverse transcription-PCR. An internal BglII/NdeI fragment

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in our laboratory (13). The mAb against human integrin GRGDSP and GRGESP were obtained from Iwaki Glass (Chiba, Japan). Radish peroxidase-conjugated goat anti-rabbit IgG were from Cappel. Goat anti-mouse IgG and horse-comple Freunds polyclonal antibody against human FN was raised in rabbits by re-
was purchased from Chemicon International, Inc. (Temecula, CA). A
Alexa Fluor™ 488-conjugated goat anti-mouse IgG and Alexa Fluor™
3.7% paraformaldehyde, and then double-stained with mouse anti-
rFN/Cm), pAI70F2mono, which encodes the C-terminal 37-kDa region
yielding the expression vector pAIFNCm, which encodes rFN/Cm.
1017-bp fragment was ligated to ApaI/NspV-cleaved pBluescript contain-
ing 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.

Antibodies and Peptides—Monoclonal antibodies (mAbs) against hu-
man FN (136H, 119A, and 17E) and hamster FN (YFN3 and 3C12) were es-

ed in our laboratory by fusion of SP2 myeloma cells with the

Cell Culture, DNA Transfection, and Selection of Stable Transformed Cells—Two adenosine kinase-deficient CHO cell lines (CHO DG44 cells) were transfectioned into CHO DG44 cells together with pGEMSVdhfr, which

Purification of Recombinant FNs and Immunoblot Analysis—CHO transfectants overexpressing recombinant FNs were maintained in

Functional Characterization of Cartilage Fibronectin

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).
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 \( \approx 220 \) 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. 2A, 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 \( \approx 500 \)-kDa region, indicative of their dimeric nature (Fig. 2A, 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. 2B, 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. 2B, 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. 3A). 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. 3B). The concentration of FN in the coating solution required to attain the maximal level of cell spreading was \( \approx 10 \) nM for rFN/C and rFN/Cm, but \( \approx 20 \) 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. 3C). 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...
with function-blocking mAbs against the integrin \( \alpha_5 \) and \( \beta_1 \) subunits, but not with those against integrin \( \alpha_6 \beta_1 \) (Fig. 4A). Consistent with the role of integrin \( \alpha_6 \beta_1 \) 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 mM GRGDSP peptide (Fig. 4B).

**Decreased Affinity of Integrin \( \alpha_6 \beta_1 \) 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 \( \alpha_6 \beta_1 \) 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 \( \alpha_6 \beta_1 \). To explore this possibility, integrin \( \alpha_6 \beta_1 \) was purified from human placenta and reconstituted into [\(^3\)H]phosphatidyicholine-containing liposomes to determine its binding activity for various recombinant FNs by solid-phase integrin-containing liposome binding assays. We found that integrin \( \alpha_6 \beta_1 \)-containing liposomes bound less avidly to rFN/O than to rFN/Cm or rFN/C (Fig. 5). The difference in the integrin \( \alpha_6 \beta_1 \)-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 \( \alpha_6 \beta_1 \)-containing liposomes to recombinant FNs was completely blocked by the anti-integrin \( \alpha_5 \) 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 \( \alpha_6 \beta_1 \).

**Cartilage-type FN Binds Strongly to Chondroitin Sulfate E—**

Not only integrin \( \alpha_6 \beta_1 \), 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 divergent 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 coassemble 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 \( \alpha_6 \beta_1 \)-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.”
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
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 α5β1. Relevant to this possibility is the previous observation that demcinectin exhibits higher binding affinity for CS compared with intact FN (29). Enhanced CS binding of demcinectin 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 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 reduction of its FN matrix-assembling activity (19). The in-

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