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Versican Interacts with Fibrillin-1 and Links Extracellular Microfibrils to Other Connective Tissue Networks*

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Fibrillin-containing microfibrils are polymeric structures that are difficult to extract from connective tissues. Proteolytic digestion of tissues has been utilized to release microfibrils for study. Few of the molecules that connect microfibrils to other elements in the matrix have been identified. In this study, electron microscopic immunolocalization of anti-versican antibodies in tissues and in extracted microfibrils demonstrated that the C-terminal region of versican is found associated with fibrillin microfibrils. Extraction of microfibrils followed by treatment of microfibrils under dissociating conditions suggested that the versican C terminus is covalently bound to microfibrils. Binding assays using recombinant fibrillin-1 polypeptides and recombinant lectican lectin domains indicated that the versican lectin domain binds to specific fibrillin-1 polypeptides. The versican lectin domain also bound to molecules comigrating with authentic fibrillin-1 monomers in an assay using cell culture medium. In assays using microfibrils, the versican lectin domain demonstrated preferential binding compared with other lecticans. Binding was calcium-dependent. The binding site for versican in microfibrils is most likely within a region of fibrillin-1 between calcium-binding epidermal growth factor-like domains 11 and 21. Human mutations in this region can result in severe forms of the Marfan syndrome ("neonatal" Marfan syndrome). The connection between versican and fibrillin microfibrils may be functionally significant, particularly in cardiovascular tissues.

Fibrillin-containing microfibrils are recognizable structural elements found throughout various connective tissues, including skin, blood vessels, perichondrium, tendons, cartilage, lung, kidney, and the ocular ciliary zonule (1). Microfibrils are present in tissues both as independent ultrastructural entities and as components of elastic fibers. Polymers of fibrillin-1 and fibrillin-2 are thought to constitute the basic structure of microfibrils (2–4). The importance of the fibrillins to the structural and functional properties of connective tissues has been demonstrated by the finding that mutations in FBN1 and FBN2 result in heritable disorders of connective tissue (5, 6).

Several extracellular matrix (ECM[1]) molecules, including MAGP-1 (7), vitronectin and amyloid P component (8), emilin (9), fibulin-1 (10), fibulin-2 (11), LTBP-1 (12), LTBP-2 (13), AMP (14), type XVI collagen (15), endostatin (16), and versican (17), have been immunolocalized to elastic fibers. However, the relationship of each of these molecules to fibrillin, to elastin, or to each other is not well understood. It is not known which molecules contribute (along with fibrillin) to the basic structure of microfibrils, which ones bridge components of elastic fibers, and which ones help to stabilize interactions between microfibrils/elastic fibers and the surrounding ECM.

High affinity binding (K_d = 56 nM) between fibrillin-1 and fibulin-2 has been demonstrated (11). However, tissue distribution studies have shown that fibulin-2 is located on some microfibrils, but not all, suggesting that the function of fibulin-2 may be to connect fibrillin-1 to elastic fiber components in a tissue-specific manner (11). Indeed, fibulin-2 was shown to bind to tropoelastin with high affinity (18).

In contrast to fibulin-2, versican displays a ubiquitous distribution in connective tissues. In skin, the staining pattern of versican is similar to that of the elastic fiber network, composed of mature elastic fibers running parallel to the epidermis and extending upwards as elaunin and oxytalan fibrils toward the dermal-epidermal junction (17). In other tissues as well, immunostaining of versican resembles elastic fiber stains (19).

Versican, also called PG-M, was originally characterized as a large chondroitin sulfate proteoglycan expressed in prechondrogenic mesenchymal condensations in developing chick limb buds (20, 21). In the ECM of certain malignant tumors, versican is also characteristically expressed (22, 23) as a component of a hyaluronan-rich matrix. In denser connective tissues such as the dermis and aorta, versican is deposited with aging (24, 25).

Based on sequence alignments, versican is a member of the lectican family of proteoglycans (26), which includes versican (27, 28), aggrecan (29), neurocan (30), and brevican (31). These proteoglycans have common features such as a hyaluronan-binding domain at the N terminus and epidermal growth factor (EGF)[1]-like, C-type lectin-like, and complement regulatory protein-like domains at the C terminus. They are highly similar in

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[1] The abbreviations used are: ECM, extracellular matrix; EGF, epidermal growth factor; mAb, monoclonal antibody; TBS, Tris-buffered saline; cbEGF, calcium-binding epidermal growth factor.

primary structure, with 40–60% identical amino acid residues. However, the tissue distributions and proposed functions are distinctive among these family members. In contrast to the ubiquitous tissue distribution of versican, aggrecan is expressed only in cartilage, whereas neurocan and brevican are specific to brain.

The investigations presented here were undertaken to determine whether versican can be found associated with specific ultrastructural elements and whether versican interacts with fibrillin-1. Results were obtained that provide new insights into the structure of fibrillin microfibrils and how matrix molecules interact to anchor fibrous elements into the amorphous ground substance.

EXPERIMENTAL PROCEDURES

Antibodies—Mouse monoclonal antibody (mAb) 2B1, raised against the large proteoglycan isolated from a yolk sac tumor, was obtained from Seikagaku Corp. (Tokyo, Japan) (32). This antibody has been shown to recognize all splicing variants of human PG-M/versican (22). Recently, the epitope for mAb 2B1 has been located within the C-terminal half of versican (V3) (33).[2] mAb 2B1 does not cross-react with fibrillin. Anti-fibrillin-1 mAbs 69, 26, and 201 have been previously characterized (1–3, 34). Antiserum specific for fibulin-2 was a gift of Dr. Rupert Timpl and has been previously characterized (35).

Isolation and Biochemical Characterization of Microfibrils—Microfibrils were extracted from human fetal membranes. Fetal membranes obtained from 13 normal placentas were minced in a Polytron homogenizer and extensively washed with water (by repeated centrifugation, followed by resuspension of the pellet) and with 50 mM Tris-HCl (pH 7.5) containing 1 M NaCl. All procedures were performed with precooled solutions at 4 °C. The pellet was finally suspended in 1 liter of 50 mM Tris-HCl (pH 7.5) containing 10 mM $CaCl_2$, 2 mM *N*-ethylmaleimide, and 1 mM phenylmethylsulfonyl fluoride. Then, 120 ml of the suspension was digested with 0.11 mg/ml highly purified collagenase (Worthington) in the presence of diisopropyl fluorophosphate (1 μl/ml; Sigma) at room temperature for 24 h and centrifuged at 38,000 $\times$ *g* for 30 min. The pellet was resuspended in 30 ml of the buffer described above, further digested with crude collagenase (0.66 mg/ml; type 1A, Sigma) in the presence of diisopropyl fluorophosphate (1 μl/ml) at room temperature for 24 h, and then centrifuged twice at 38,000 $\times$ *g* for 30 min. The supernatant was digested with 0.5 mg/ml DNase I (Roche Molecular Biochemicals) at room temperature for 3 h. 20-ml aliquots were fractionated on a Sepharose CL-2B molecular sieve column (total volume of 270 ml; Amersham Biosciences, Inc.) equilibrated in 50 mM Tris-HCl (pH 7.5) containing 10 mM $CaCl_2$ at a flow rate of 0.3 ml/min. The fractions were collected every 3.6 ml (12 min/tube).

The fractions that eluted in the void volume (see Fig. 2*A*, *bar*) were analyzed by electron microscopy after rotary shadowing. SDS-PAGE (5% acrylamide) of the void volume fractions was performed using 10 μg of total protein/well under nonreducing conditions, after samples were treated with or without chondroitinase ABC (36). Dot blot analysis was performed using a nitrocellulose filter spotted with 2.5 μl from every other fraction. The blot was blocked with 5% nonfat milk in Tris-buffered saline (TBS) for 1 h and then incubated with mAb 2B1 (diluted 1:3000) or mAb 69 or 26 (each diluted 1:400) in TBS. After washing with 0.05% Tween 20 in TBS, the blots were incubated with peroxidase-conjugated anti-mouse IgG (Sigma). The blots were developed with chemiluminescent substrate (SuperSignal, Pierce) according to the instructions of the manufacturer.

To determine whether aggregates containing hyaluronan and versican were coeluting with fibrillin microfibrils, microfibril fractions (see Fig. 2*A*, *bar*) were pooled, dialyzed against 20 mM sodium acetate (pH 5.8) containing 0.15 M NaCl, and then digested with 8.45 units/ml hyaluronidase (from *Streptomyces hyalurolyticus*; Sigma) at room temperature for 24 h. The microfibril sample was rechromatographed on Sepharose CL-2B, and fractions were analyzed by dot blotting with mAb 2B1 and anti-fibrillin antibodies. In addition, the supernatant from the tissues (extracted with crude collagenase and digested with DNase I) was dialyzed against 20 mM sodium acetate (pH 5.8) containing 0.15 M NaCl, treated with 8.45 units/ml hyaluronidase at room temperature for 24 h, and applied to the Sepharose CL-2B column. Fractions were analyzed by dot blotting.

In addition, fractions containing microfibrils (see Fig. 2*A*, *bar*) were pooled and dialyzed against 4 M guanidine HCl and 50 mM Tris-HCl (pH 7.5) using a membrane cutoff of M_r 10,000. Then, 10 ml of the dialyzed sample was concentrated using a Centriplus filtration unit (membrane cutoff of M_r 10,000; Amicon, Inc.) to 6 ml and subjected to molecular sieve chromatography under dissociating conditions. A Sepharose CL-2B column (total volume of 90 ml) was equilibrated in 4 M guanidine HCl and 50 mM Tris-HCl (pH 7.5), and the sample was applied at a flow rate of 0.1 ml/min. Protein concentrations were determined using a BCA protein assay kit (Pierce) with bovine serum albumin as the standard. Dot blot analysis was performed as described above.

Rotary Shadowing Electron Microscopy—Samples containing microfibrils were dialyzed against H_2O and rotary-shadowed as described previously (37).

Immunogold Negative Staining Electron Microscopy—Microfibrils obtained after sieve chromatography (see Fig. 2, *A* and *B*) were used. Microfibrils were incubated with mAb 2B1 and then further incubated with secondary antibody (goat anti-mouse IgG conjugated to 5-nm gold particles). After incubation, the samples were deposited onto carbon-coated 600-mesh grids and contrasted with 2% (w/v) phosphotungstic acid (pH 7.0). Controls included samples in which the primary antibody was omitted or in which primary antibodies of alternate specificity were used.

Localization in Tissues—Fresh neonatal foreskin was immunolabeled as described previously (38). The tissue was rinsed in phosphate-buffered saline and then submerged overnight with mAb 2B1 diluted 1:5 in phosphate-buffered saline. Following an extensive rinse in phosphate-buffered saline, the tissue was submerged overnight with 5-nm colloidal gold-conjugated goat anti-mouse IgG (Amersham Biosciences, Inc.), rinsed extensively in phosphate-buffered saline and 0.1 M cacodylate buffer, fixed, dehydrated, and embedded in Spurrs epoxy. 90-nm-thick sections, which included reticular dermis, were stained with uranyl acetate and lead citrate and examined with a Philips EM410 transmission electron microscope. Controls included samples in which the primary antibody was omitted or in which primary antibodies of alternate specificity were used.

Expression and Purification of Peptides—Recombinant fibrillin-1 polypeptides rF6 (C-terminal half), rF11 (N-terminal half), rF18 (3), rF23 (11), rF31 (39), rF38 (40), and rF45 (41) have been described. These polypeptides have been characterized by sequence analysis, calcium binding, reactivity to monoclonal antibodies, rotary shadowing electron microscopy, hydrodynamic methods, and CD spectroscopy. By all analytical tests employed, these recombinant polypeptides appear to be properly folded.

The production of recombinant lectin domains of lecticans has been reported (42–44), and the expression of recombinant lectin domain-alkaline phosphatase fusion proteins is described in detail elsewhere (45). To produce the fusion proteins, a secreted alkaline phosphatase module was inserted in frame C-terminal to the lectin domains, followed by a hexahistidine tag.

Binding Assays—Affinity measurements between lectin-like domains of proteoglycans and fibrillin-1 polypeptides were obtained using surface plasmon resonance. Purified recombinant lectin-like domains were diluted in 10 mM sodium acetate (pH 4.0) and coupled to a BIAcore CM5 sensor chip, resulting in immobilization levels of 1700–2000 response units. Recombinant fibrillin-1 polypeptides (1.9–31.25 nM) were flowed over the lectin surface and a blank surface at 35 μl/min. The running buffer was 10 mM HEPES, 2 mM $CaCl_2$, and 0.005% surfactant P20. The surface was regenerated by injection of 20 mM EDTA in the running buffer after each cycle. After subtraction of the blank surface curves, the association and dissociation rate constants for each lectin/peptide combination were determined using a simultaneous global curve fit to the response traces (BIAevaluation Version 3.0). Mass transfer limitations were apparent neither in the derivative of association curves nor in control experiments with injections of a constant concentration of analyte at different flow rates.

Blot overlay assays were performed as described previously (11) using serum-free conditioned medium from human cell cultures. Normal dermal fibroblasts were obtained from explant cultures of neonatal foreskin. A204 rhabdomyosarcoma cells were obtained from American Type Culture Collection. Cells were grown to confluency in T-75 flasks and incubated for 24 h in 7 ml of serum-free medium. Media samples were harvested, and phenylmethylsulfonyl fluoride was added to 2 mM. The dermal fibroblast cell layer was scraped up and extracted in 7 ml of TBS, 10 mM EDTA, and 2 mM phenylmethylsulfonyl fluoride. 1 ml of conditioned medium or cell layer was precipitated by trichloroacetic acid, resolved under nonreducing conditions on 4.5% SDS-PAGE, and transferred to nitrocellulose membrane. Recombinant versican lectin domain-alkaline phosphatase fusion proteins (1 μg/ml) in TBS contain-

[2] Tamayuki Shinomura, personal communication.

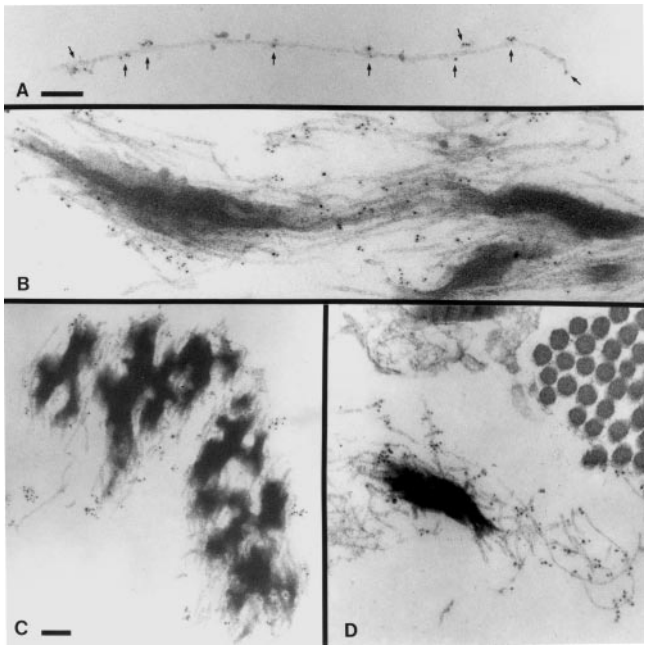

FIG. 1. **Immunoelectron microscopic localization of versican to microfibrils.** In human neonatal skin, anti-versican mAb 2B1 directed gold labeling to individual microfibrils (*A*) and to microfibrils associated with amorphous elastin cores (*B–D*). Gold labeling events did not display periodicities. *Bars* = 100 nm.

ing 5 mM $CaCl_2$ were incubated with the blots at 4 °C overnight. After washing three times with 0.05% Tween 20 in TBS, color development was achieved using alkaline phosphatase substrate (1-Step nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate, Pierce). Western blot analysis was performed with polyclonal antibody 1035, specific for fibulin-2 (35), and polyclonal antibody 9543, specific for fibrillin-1,[3] both diluted 1:2000 in TBS and 0.05% Tween 20.

Binding between lectin-like domains and isolated microfibrils was analyzed in solid-phase assays. Void volume fractions consisting of microfibrils were obtained from the non-dissociating Sepharose CL-2B column and used to coat 96-well plates (10 μg/ml, 100 μl/well) in 15 mM Na_2CO_3 and 35 mM $NaHCO_3$ (pH 9.2) overnight at 4 °C. Coated wells were then blocked with 0.3% bovine serum albumin in 50 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl at room temperature for 1 h. Recombinant lectin domains (maximum of 1 μM) were serially diluted 1:3 in 50 mM Tris-HCl (pH 7.4), 0.15 M NaCl, and 2 mM $CaCl_2$ or 5 mM EDTA. The lectin domains were incubated with the immobilized microfibrils at room temperature for 3 h, and then the wells were washed three times with 0.05% Tween 20 in TBS containing 2 mM $CaCl_2$. Color development was achieved by incubation with the alkaline phosphatase substrate *p*-nitrophenyl phosphate (tablet sets, Sigma) for 20 min. The absorbance at 405 nm was measured after the reaction was stopped with EDTA.

RESULTS

Immunolocalization of Versican to Microfibrils—Light microscopic immunohistochemistry previously demonstrated an elastic fiber staining pattern for versican in human skin (17). Therefore, to determine whether versican is associated with fibrillin microfibrils, electron microscopic immunolocalization was performed. When neonatal skin was labeled with anti-versican mAb 2B1, antibody-directed gold labeling was observed decorating both microfibrils that were independent of amorphous elastin (Fig. 1*A*) and elastin-associated microfibrils (Fig. 1, *B–D*). Although labeling was found all along microfibrils (Fig. 1*A*) and throughout microfibril bundles in elastic fibers (Fig. 1, *B–D*), a dense periodic labeling similar to that directed by fibrillin antibodies (1, 3) was not evident.

Extracted Microfibrils Contain Versican—When human fetal membranes were first digested with purified collagenase and the supernatant was applied to the molecular sieve column, no peak was observed in the void volume (data not shown), indicating that microfibrils were not released from tissues by specific degradation of collagen. However, when the pellet was further digested with crude collagenase and the supernatant was applied to the sieve column, void volume fractions (Fig. 2*A*, *bar*) contained microfibrils of various lengths (Fig. 2*B*). No other structural macromolecular aggregates (*e.g.* type VI collagen filaments, proteoglycan complexes) were apparent in low and high magnification fields (Fig. 2*B*). Dot blot analysis of sieve column fractions confirmed the presence of fibrillin-1 in the void volume microfibril-containing fractions (data not shown).

Dot blot analysis of all fractions from the column using mAb 2B1 demonstrated that the void volume fractions contained most, if not all, of the extracted mAb 2B1-reactive versican (Fig. 2*A*). Because monomeric PG-M/versican elutes in the middle of Sepharose CL-2B-included fractions (20), these results, together with immunolocalization of versican to microfibrils (Fig. 1), suggested that versican might be associated with the microfibrils present in the void volume fractions. Alternatively, versican could be associated with high molecular mass aggregates other than fibrillin microfibrils present, but not apparent, in these fractions. To exclude the presence of microfibril-independent versican/hyaluronan aggregates, collagenase-digested samples were further subjected to hyaluronidase digestion. Both microfibril fractions and unfractionated samples were subjected to hyaluronidase digestion and chromatographed using Sepharose CL-2B. Versican mAb 2B1 reactivity remained primarily in the microfibril fractions (data not shown).

To determine whether monomeric versican was present in the high molecular mass microfibril fractions, these fractions (Fig. 2*A*, *bar*) were treated with chondroitinase ABC and subjected to SDS-PAGE and immunoblotting. After chondroitinase treatment and dissociation of the sample in SDS, most of the Coomassie Blue-stained materials stayed at the top of the stacking gel, indicating that most of the protein present in the sample remained in large non-dissociable aggregates. Even sensitive immunoblotting failed to reveal the versican monomer or mAb 2B1-reactive fragments (Fig. 2*C*), suggesting that the immunoreactive versican present in the sample was unable to enter the running gel.

To further test whether versican could be separated from the microfibrils, microfibril fractions from the sieve column (Fig. 2*A*) were rechromatographed in buffer containing 4 M guanidine. These dissociating conditions resulted in the separation of some noncovalently associated proteins. However, fibrillin-1 (data not shown) and versican immunoreactivities were still detected in the microfibril fractions (Fig. 3). Collectively, these data indicate that versican is bound to macromolecules other than hyaluronan.

Microfibrils were immunolabeled with mAb 2B1 to demonstrate specific association of versican with beaded strings of microfibrils. In negatively stained preparations, mAb 2B1 directed gold labeling to the beaded string structures (Fig. 4). Because the bottle-brush structure of monomeric PG-M/versican (46) was not clearly apparent after rotary shadowing electron microscopy of microfibrils (Fig. 2*B*), perhaps only a portion of versican (containing the mAb 2B1 epitope) remained undegraded and associated with microfibrils following digestion of tissues with crude collagenase.

Versican Lectin Domain Binds to Fibrillin-1 and to Microfibrils—The epitope of mAb 2B1 is within the carboxyl-terminal region of versican (Fig. 5) (22).[2] Because this portion of versican

[3] N. L. Charbonneau, B. J. Dzamba, R. N. Ono, D. R. Keene, D. P. Reinhardt, and L. Y. Sakai, manuscript in preparation.

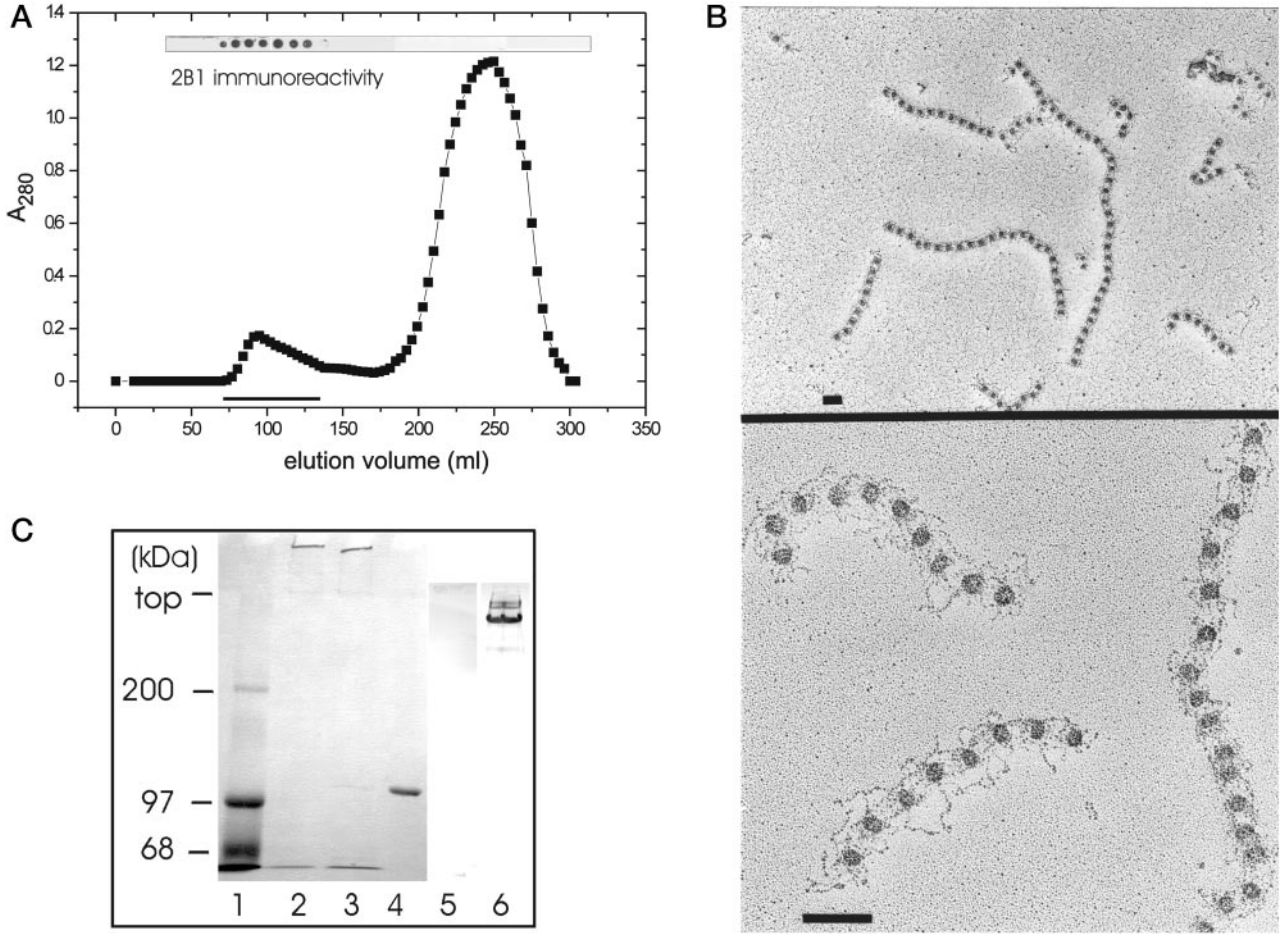

FIG. 2. **Sieve chromatography of crude collagenase-digested fetal membranes and characterization of microfibril-containing fractions.** *A*, proteins solubilized by digestion with crude collagenase were separated by size on a Sepharose CL-2B column (total volume of 270 ml). Every other fraction was tested by dot blot analysis using anti-versican mAb 2B1 (*inset*). Fractions eluting in the peak closest to the void volume (*bar*) were positive in dot blots; all other fractions were mAb 2B1-negative. *B*, the fractions indicated by the *bar* in *A* were analyzed by rotary shadowing electron microscopy, which revealed the presence of multiple strings of beaded microfibrils. *Bars* = 100 nm. *C*, when the microfibril-containing fractions were analyzed by SDS-PAGE (5% acrylamide) followed by Coomassie Blue staining, most of the protein remained at the top of the stacking gel (*lane 2*), even after the sample was treated with chondroitinase ABC (*lane 3*). *Lane 1* contains molecular mass markers; *lane 2* contains 10 μg of microfibril fractions; *lane 3* contains 10 μg of microfibril fractions incubated with chondroitinase ABC; and *lane 4* contains chondroitinase ABC. Immunoblotting of the chondroitinase-treated microfibril fractions did not reveal mAb 2B1-reactive versican or versican fragments (*lane 5*). For the immunoblots, the running gel (but not the stacking gel) was transferred to nitrocellulose. *Lane 6* is an immunoblot of chondroitinase-treated fibroblast medium, demonstrating mAb 2B1-positive versican.

was immunolocalized to microfibrils (Figs. 1 and 4) and the lectin domain has been shown to mediate interactions with other extracellular matrix components (42–45), recombinant lectin domains from versican and other lecticans were tested for interactions with fibrillin-1 peptides (Fig. 5).

Surface plasmon resonance analysis was performed to determine kinetic rates in real time and to map the binding site for versican on fibrillin-1 (Fig. 6). Table I summarizes the results obtained using lectin domains from versican, aggrecan, brevican, and neurocan and various fibrillin-1 polypeptides. All lectin domains bound to rF11, the N-terminal half of fibrillin-1, but failed to bind to rF6, the C-terminal half of fibrillin-1. In this assay, rF23, a smaller polypeptide containing the N terminus and the first nine domains of fibrillin-1, also bound to all lectin domains. However, rF31 and rF38, subdomains of rF23, did not bind to the lectin domains, suggesting that binding to rF23 may be mediated by the N terminus and first EGF domain of fibrillin-1. It cannot, however, be excluded that the rF23 binding site resides at the border between rF31 and rF38 (cbEGF domains 1 and 2) (Fig. 5).

In contrast, rF18, containing the longest stretch of cbEGF-like domains in fibrillin-1 as well as the two preceding domains, bound to versican and aggrecan domains, but not to brevican and neurocan. The binding affinity between versican and rF18 was 7-fold higher than that between aggrecan and rF18. Because rF45 and rF6, which partially overlap rF18, did not bind the lectin domains, it is likely that the fibrillin-1 binding site for versican resides somewhere between cbEGF domains 11 and 21. The interactions observed were calcium-dependent; bound ligands dissociated when calcium ions were chelated with EDTA. When the recombinant fibrillin polypeptides were injected over the lectin surface with EDTA in the running buffer, no binding was observed (data not shown). In blot overlay assays, the versican lectin domain bound to rF18, but not to rF23, although equal molar quantities of polypeptides were tested (data not shown).

To confirm these binding activities obtained using recombinant polypeptides, authentic fibrillin-1 monomers were tested using a blot overlay assay. Proteins present in conditioned serum-free medium from various human cell cultures were separated by SDS-PAGE and transferred to nitrocellulose membranes. To distinguish between fibrillin-1 and fibulin-2, cell cultures synthesizing both proteins (normal skin fibroblasts) and cell lines making predominantly fibrillin-1 (A204) were utilized for the versican blot overlay. The blot

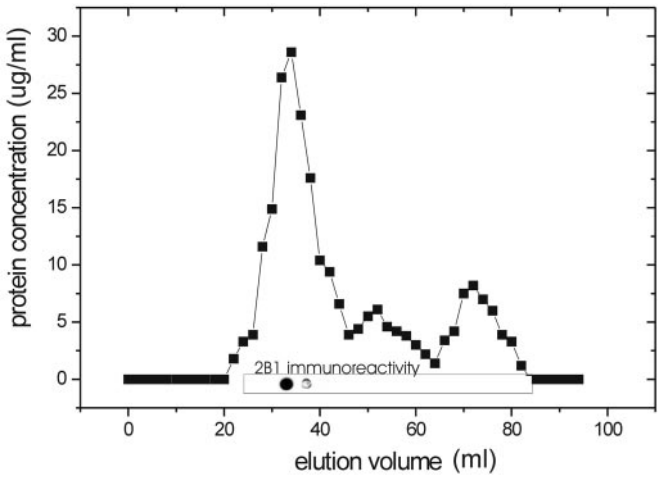

FIG. 3. **Rechromatography of the microfibril-containing fractions from Fig. 2 under dissociating conditions does not separate mAb 2B1-positive versican from the microfibrils.** The microfibril-containing fractions (Fig. 2*A*, *bar*) were dialyzed against 4 M guanidine HCl and 50 mM Tris-HCl (pH 7.5) and then applied to a Sepharose CL-2B column (total volume of 90 ml) equilibrated in the same buffer. Dot blot analysis was performed with aliquots from every other fraction (*inset*). mAb 2B1-positive material remained in the microfibril-containing peak.

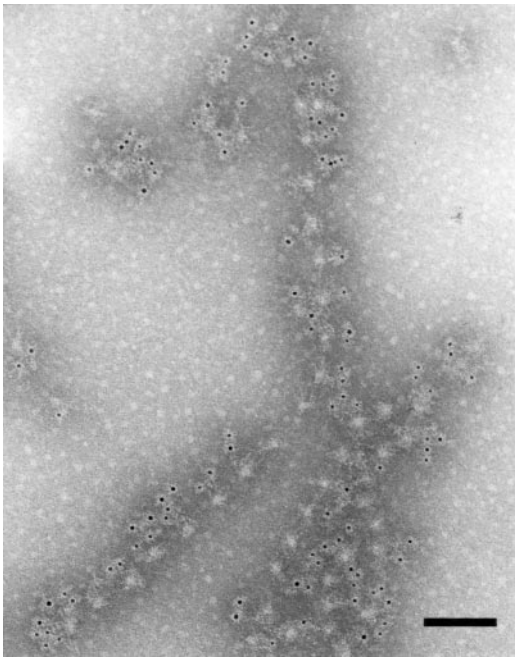

FIG. 4. **Immunogold labeling of microfibrils using anti-versican mAb 2B1.** mAb 2B1 directed gold labeling to beaded microfibrils. Antibody-bound preparations of microfibrils (obtained as shown in Fig. 2*A* and seen in Fig. 2*B*) were visualized by negative staining. *Bar* = 100 nm.

was incubated with the versican lectin domain in buffer containing calcium. The versican lectin domain was bound to a band comigrating with authentic fibrillin-1 monomers secreted by the cell lines (Fig. 7). In addition, the versican lectin domain was bound to a band comigrating with fibulin-2. In fibroblast medium, two additional bands were also bound by the versican lectin domain. The high molecular mass band at the top of the running gel comigrated with tenascin C; the lower molecular mass band comigrated with fibulin-1. Disulfide-linked fibulin-2 dimers migrated more slowly on gels compared with fibrillin-1 monomers (Fig. 7) and are preferentially secreted into the matrix of fibroblasts in culture (35). Fibrillin-1 is rapidly assembled in

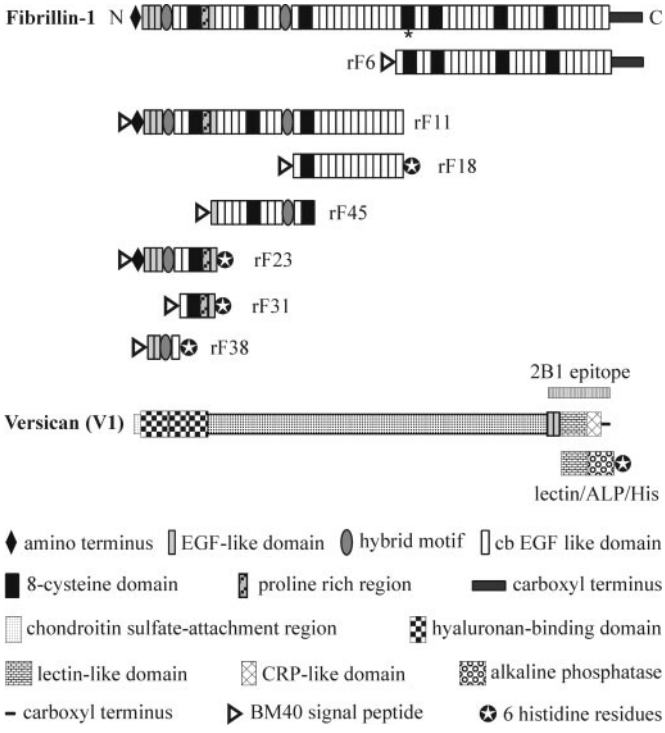

FIG. 5. **Schematic drawings of fibrillin-1 and versican.** The recombinant fibrillin-1 polypeptides used in this study are mapped below the schematic of fibrillin-1. The lectin domain fusion protein used in this study is shown below versican. The mAb 2B1 epitope, marked above the C-terminal end of versican, is likely to be contained in the EGF domains adjacent to the lectin domain. *ALP*, alkaline phosphatase; *CRP*, complement regulatory protein.

the matrix into multimers (40), so only small amounts of monomeric fibrillin-1 are present in cell layer extracts.[4]

To further specify interactions between lecticans and fibrillin-1, soluble lectin domains were tested in solid-phase assays with immobilized isolated microfibrils (Fig. 8). In this assay, the versican lectin domain preferentially bound to the microfibrils. In contrast, the brevican and neurocan lectin domains, which exhibited binding to rF23, but not to rF18, did not bind to the microfibrils. The aggrecan lectin domain, which demonstrated lower affinity for rF18 compared with the versican domain in the surface plasmon resonance measurements, bound only weakly to the microfibrils. When calcium was replaced by EDTA in the buffer, the binding of the versican lectin domain was abolished. Color development by these lectin domain-alkaline phosphatase fusion proteins was equal (data not shown).

DISCUSSION

The connective tissue provides mechanical support and functional integrity to the various organ systems of the body. Because the connective tissue is designed to hold organs together, isolation of the constitutive molecules and macromolecular complexes has usually involved disruptive or degradative protocols. Fibrillin microfibrils have been extracted from tissues by dissociating and non-dissociating degradative methods and visualized as beaded strings by rotary shadowing and electron microscopy (38, 47). Digests of tissue using crude preparations of collagenase followed by molecular sieve chromatography have been commonly used to obtain beaded string fibrillin microfibrils (48).

[4] B. J. Dzamba, D. R. Keene, Z. Isogai, N. L. Charbonneau, N. K. Juruskova, M. Simon, and L. Y. Sakai, (2001) *J. Invest. Dermatol.* **117,** 1612–1620.

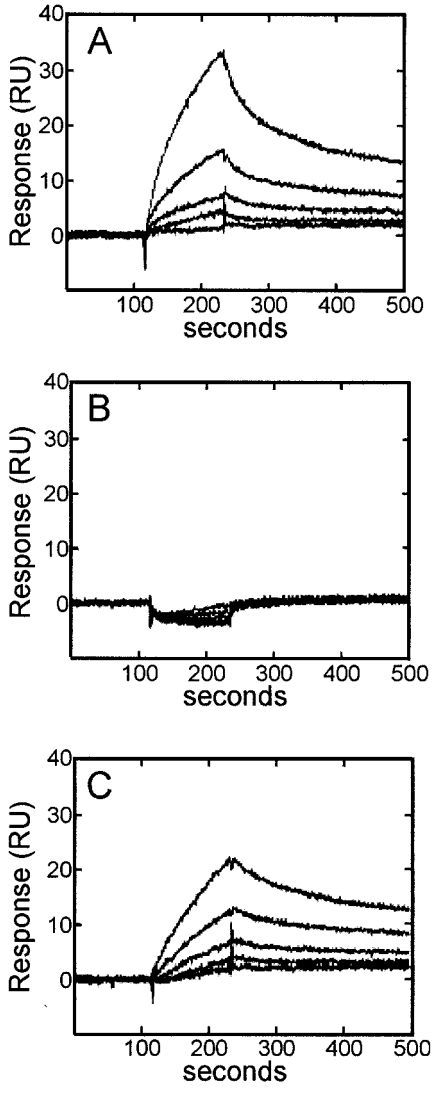

FIG. 6. **Representative sensograms of surface plasmon resonance measurements of lectin domain/rF18 interactions.** Recombinant fibrillin-1 polypeptide rF18 was injected over immobilized recombinant proteoglycan C-type lectin domains in a BIAcore 2000 instrument. *A–C*, aggrecan, brevican, and versican recombinant proteoglycan C-type lectin domains, respectively. Injection started at 115 s and ended at 235 s. The different curves in each panel show response traces for different rF18 concentrations (1.9–31.3 nM). The rF18 polypeptide showed clear binding to aggrecan (*A*) and versican (*C*) C-type lectin domains. No binding was seen to brevican (*B*) or neurocan C-type lectin domains. In control experiments, all four C-type lectin domain surfaces bound tenascin R (not shown). Bound fibrillin peptide rF18 was rapidly and completely removed through injection of EDTA (not shown). The binding buffer contained 2 mM $CaCl_2$. *RU*, response units.

In the investigations described here, purified bacterial collagenase was used first to attempt to liberate microfibrils from tissues. This procedure did not yield beaded string microfibrils, suggesting that stabilization of microfibrils within the connective tissue space is not accomplished by simple entrapment of microfibrils between collagen bundles. Further digestion of the tissue with crude collagenase (which contains other undefined proteases) was required to liberate beaded strings of microfibrils.

Evidence provided in this study suggests that versican is one protein that binds to microfibrils and may stabilize microfibrils within the connective tissue space through additional interactions. First, electron microscopic immunolocalization demonstrated that versican is specifically associated with microfibrils throughout the elastic fiber network of the dermis. In contrast to amyloid P component, which is not present on elastic fibers in the dermis of young individuals (8), versican was immunolocalized here in neonatal dermis and was colocalized with fibrillin-1 in all fetal tissues examined (skin, skeletal muscle, tendon, cartilage, and vasculature) (data not shown). However, because immunolabeling of microfibrils was relatively sparse and therefore did not suggest periodicity, we conclude that versican does not serve the same backbone structural function as fibrillin.

Both fibulin-1 (44) and fibulin-2 (45) are ligands for the versican lectin domain, and fibulin-2 is a ligand for fibrillins (11). However, fibulin-2 is preferentially localized to the elastin/microfibril interface in some tissues, but not in others (11), and fibulin-1 is found in amorphous elastin cores in old skin (10). In contrast to the limited immunolocalization and distribution patterns of the fibulins, the ubiquitous tissue distribution and immunolocalization of versican along the microfibrils suggest that versican can interact directly with fibrillin-1 *in vivo* and that fibulins are not required to mediate this interaction. In human amnion, staining with antibodies to fibulin-2 was negative (data not shown), and preparations of microfibrils used for investigating interactions with versican did not contain fibulin-2 when tested by enzyme-linked immunosorbent assay with anti-fibulin-2 antibodies (data not shown). However, it remains possible that the fibulins may form a bridge between versican and fibrillin molecules in some cases.

Second, the C-terminal end of versican was identified on extracted beaded string microfibrils by immunolabeling of the beaded strings. However, the versican proteoglycan moiety was not visualized on beaded strings after rotary shadowing. We interpret these results to indicate that the chondroitin sulfate attachment domain of versican is degraded during the extraction procedure using crude collagenase, whereas the C-terminal end is resistant to these conditions. Results presented here also suggest that the C-terminal end of versican may be covalently linked to microfibrils because versican and microfibrils coeluted in the void volume in dissociating Sepharose CL-2B molecular sieve chromatography. The finding that 4 M guanidine did not dissociate versican from the microfibrils indicates covalently cross-linked versican complexes, but whether versican is actually cross-linked to fibrillin or to other molecules remains to be determined.

Finally, binding studies using recombinant polypeptides as well as authentic fibrillin-1 monomers demonstrated a high affinity interaction between the versican lectin domain and fibrillin-1. Surface plasmon resonance assays indicated K_d values in the low nanomolar range. The major site in fibrillin-1 for this interaction was determined to reside between cbEGF domains 11 and 21, contained in rF18. It is of interest to note that the binding specificity between these lectin domains and rF18 is similar to that between lectin domains and the extracellular matrix proteins fibulin-1 (44) and fibulin-2 (45), where versican and aggrecan lectin domains also interact with cbEGF-like domains present in the fibulins. As expected for a C-type lectin and for cbEGF domains, the interactions with fibrillin-1 were calcium-dependent.

The interaction between versican and fibrillin-1 was confirmed in blot overlay assays using authentic fibrillin-1 monomers present in conditioned medium from human cell cultures. In these experiments with selected cell types, fibrillin-1 and fibulin-2 were identified as native intact ligands for the versican lectin domain. Interactions with other proteins (tenascin C and fibulin-1) were also suggested in these experiments.

Comparison with other lectican lectin domains demonstrated

TABLE I

Surface plasmon resonance affinity measurements between proteoglycan C-type lectin-like domains and fibrillin-1 polypeptides

Units are as follows: k_a = M^{-1} s^{-1} × 10^{-3}, k_d = s^{-1} × 10^{-3}, and K_d = nM. NB, no binding; ND, not determined.

Ligand	Versican			Aggrecan			Brevican			Neurocan		
	k_a	k_d	K_d	k_a	k_d	K_d	k_a	k_d	K_d	k_a	k_d	K_d
rF6	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB
rF11	323	2.1	6.5	395	3.1	7.8	133	2.66	19.9	586	1.57	2.67
rF18	286	2.02	7.1	55.3	2.75	49.7	NB	NB	NB	NB	NB	NB
rF45	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB
rF23	137	2.36	17.2	65.6	2.77	42.2	41.4	2.88	69.6	633	1.03	1.62
rF31	NB	NB	NB	NB	NB	NB	ND	ND	ND	NB	NB	NB
rF38	NB	NB	NB	NB	NB	NB	ND	ND	ND	NB	NB	NB

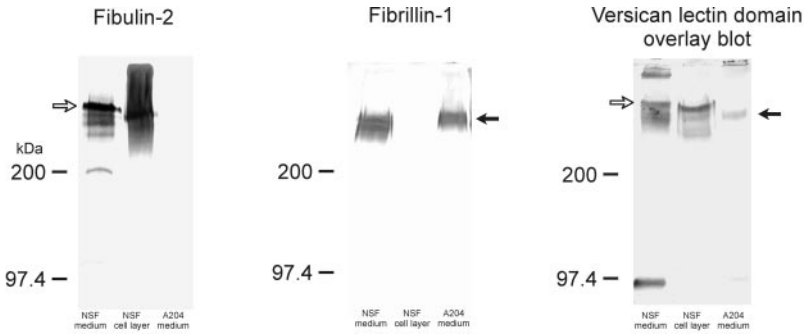

FIG. 7. **Blot overlay assay using recombinant versican lectin domain and human cell culture proteins.** Equal aliquots of conditioned media from normal skin fibroblasts (*NSF*) and A204 rhabdomyosarcoma cells and equal aliquots of normal skin fibroblast cell layer extract were resolved by 4.5% SDS-PAGE and transferred to nitrocellulose membranes. For immunoblotting and binding assays, samples were run without disulfide bond-reducing agent. Immunoblotting of the samples was performed using polyclonal antibody 1035, specific for fibulin-2, and polyclonal antibody 9543, specific for fibrillin-1. The electrophoretic position of fibulin-2 is marked with an *open arrow*, and the position of fibrillin-1 is marked with a *closed arrow*. For the blot overlay assays, recombinant versican lectin domain-alkaline phosphatase fusion proteins were used as the soluble ligand. Binding of the soluble versican lectin domain to cell culture proteins immobilized on the membranes was visualized using alkaline phosphatase substrate. The versican lectin domain was bound to proteins comigrating with fibulin-2 and fibrillin-1 and to a species migrating at ~97 kDa, which could be fibulin-1. The positions of molecular mass marker proteins are indicated.

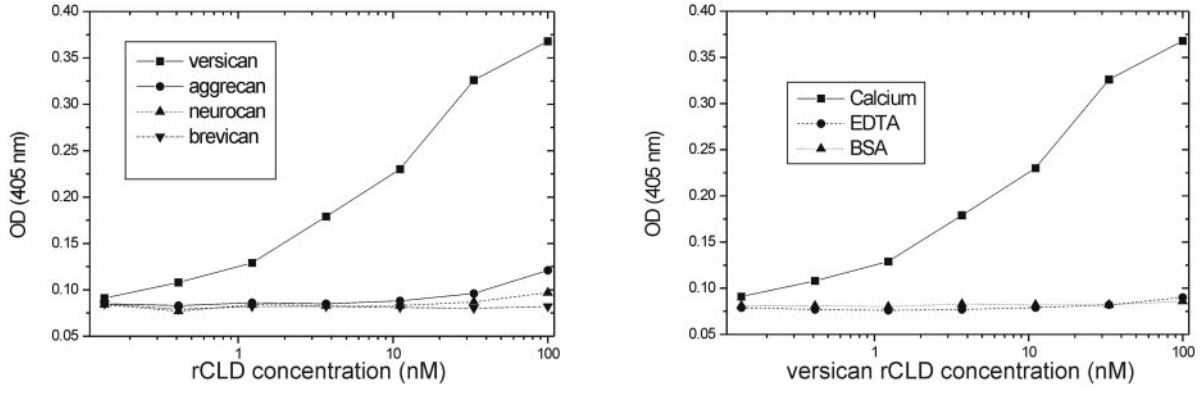

FIG. 8. **Solid-phase binding assay of proteoglycan lectin domains and isolated microfibrils.** Isolated microfibrils (shown in Fig. 2*B*) or bovine serum albumin (*BSA*) was used to coat wells of microtiter plates. Then, various concentrations of recombinant lectin domain-alkaline phosphatase fusion proteins were incubated in the presence of calcium or EDTA. Color development was achieved using alkaline phosphatase substrate. The versican lectin domain, but not the neurocan, brevican, or aggrecan lectin domain, demonstrated significant binding to microfibrils. Binding of the versican lectin domain to microfibrils was abolished in the presence of EDTA. Data points represent averages of triplicate samples. *rCLD*, recombinant proteoglycan C-type lectin domain.

specific binding of versican and aggrecan lectin domains to rF18. Despite high sequence homology between lectin domains (31), neurocan and brevican lectin domains did not bind to rF18. In contrast to rF18, rF11 and the smaller subdomain of rF11, rF23, appeared to bind all lectican lectin domains in surface plasmon resonance assays. However, in blot overlay assays, only rF18 was bound by versican (data not shown).

When isolated microfibrils from human fetal membranes were used as a substrate in solid-phase binding assays, neurocan and brevican lectin domains demonstrated no binding activity. Moreover, the versican lectin domain showed strong binding to the microfibrils, whereas the aggrecan lectin domain displayed only slight binding. These binding data are similar to those obtained from surface plasmon resonance with rF18. The results confirm that the binding sites on fibrillin-1 for the versican lectin domain are not fully occupied in tissue microfibrils, as indicated by the relatively sparse immunolabeling of microfibrils. In addition, these data suggest that binding sites for versican are exposed and accessible on microfibrils.

Taken together, the data indicate that the versican lectin domain binds to a site on fibrillin-1 between cbEGF domains 11 and 21. This site is available to versican in the polymerized form that fibrillin-1 adopts in microfibrils present in the dermis and fetal membranes. Other sites (those present in rF11/rF23) suggested as binding sites that might be utilized by other lectican lectin domains appear not to be available within the polymerized form of fibrillin-1 in fetal membrane microfibrils because only the versican lectin domain bound well to fetal

membrane microfibrils. Whether the aggrecan lectin domain preferentially binds to fibrillin-1 in cartilage microfibrils, compared with microfibrils from fetal membranes, is unknown. It is also unknown whether fibrillin-1 monomers or microfibrils exist in neural tissues and whether neurocan or brevican can bind to fibrillin-1 in these tissues. High affinity binding sites determined by surface plasmon resonance to be contained in rF11/rF23 may be utilized by other lecticans in other tissues.

Because versican contains many chondroitin sulfate chains attached to the central domain and has hyaluronan-binding capacity through interaction with the N-terminal region (27, 49), versican may connect microfibrils to a hyaluronan-rich matrix with large water-holding capacity. Hyaluronan-rich matrices are thought to confer elasticity to tissues and may be of particular importance to tissues like the aorta. The interaction between versican and fibrillin microfibrils may therefore represent a significant functional connection. Mutations in FBN1 that occur in the putative versican-binding domain (cbEGF domains 11–21) of fibrillin-1 can result in severe forms of the Marfan syndrome, including "neonatal" Marfan syndrome (50). It remains to be tested, however, whether mutations in this region can disrupt binding of fibrillin microfibrils to versican and whether destabilization of this interaction is sufficient to cause severe disease.

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Versican Interacts with Fibrillin-1 and Links Extracellular Microfibrils to Other Connective Tissue Networks

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