The Proteoglycans Aggrecan and Versican Form Networks with Fibulin-2 through Their Lectin Domain Binding* 

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Aggrecan, versican, neurocan, and brevican are important components of the extracellular matrix in various tissues. Their amino-terminal globular domains bind to hyaluronan, but the function of their carboxy-terminal globular domains has long remained elusive. A picture is now emerging where the C-type lectin motif of this domain mediates binding to other extracellular matrix proteins. We here demonstrate that aggrecan, versican, and brevican lectin domains bind fibulin-2, whereas neurocan does not. As expected for a C-type lectin, the interactions are calcium-dependent, with $K_D$ values in the nanomolar range as measured by surface plasmon resonance. Solid phase competition assays with previously identified ligands demonstrated that fibulin-2 and tenascin-R bind the same site on the proteoglycan lectin domains. Fibulin-1 has affinity for the common site on versican but may bind to a different site on the aggrecan lectin domain. By using deletion mutants, the interaction sites for aggrecan and versican lectin domains were mapped to epidermal growth factor-like repeats in domain II of fibulin-2. Affinity chromatography and solid phase assays confirmed that also native full-length aggrecan and versican bind the lectin domain ligands. Electron microscopy confirmed the mapping and demonstrated that hyaluronan-aggrecan complexes can be cross-linked by the fibrils.

The large aggregating proteoglycans referred to as lecticans (1) or hyalactans (2) are ubiquitous extracellular matrix components. The family consists of aggrecan, versican, brevican, and neurocan. Aggrecan is expressed by chondrocytes in cartilage. Versican is found in many tissues, e.g. blood vessels and demir expressed by smooth muscle cells and fibroblasts. Brevican and neurocan are found in the central nervous system, expressed by astrocytes and neuronal cells, respectively. These proteoglycans have important functions in many tissues. Aggrecan, for example, with its many glycosaminoglycan side chains and thus high fixed charge density gives rise to a pronounced osmotic swelling pressure that is crucial for the biomechanical properties of cartilage (3, 4).

The core proteins of these proteoglycans are organized with a central elongated glycosaminoglycan-carrying part of variable length, flanked by globular domains mediating interactions with other matrix molecules (5–9). The amino-terminal globular G1 domains bind to hyaluronan in interactions stabilized by link protein (10–13). The carboxy-terminal globular G3 domain consists of one or two EGF repeats, a C-type lectin-like domain (CLD), and a sushi repeat. It has recently been shown that the CLDs function in binding. So far four ligands have been identified as follows: the brain-specific extracellular matrix protein tenasin-C (14, 15), tenasin-C (16), sulfated glycolipids (17), and fibulin-1 (18).

The CLDs of the lecticans are highly conserved; the amino acid sequence of the chick versican CLD, for example, is 96% identical to the human homologue. This high degree of conservation suggests that the CLD motif of the G3 domain has important functions. We propose that one of these functions is to organize the forming hyaluronan-lectican complexes in the assembly of the extracellular matrix. This can be achieved through binding other matrix components with multiple lectican CLD-binding sites. Several of the CLD ligands identified so far are multimeric proteins as follows: tenasin-C is a dimer or trimer (19), tenasin-C is a hexamer (20, 21), and fibulin-1 may self-assemble (22, 23). In all these cases the lectican CLD binding is directed to extended rod-like stretches of the multimeric extracellular matrix molecules. These proteins are thus excellent candidates for cross-linking the hyaluronan-lectican complexes. Due to the very large size of the link protein-stabilized hyaluronan-lectican complexes, such cross-linking is probably not critical to retain the lecticans in the mature extracellular matrix. Indeed, previous studies have shown that although proteolytic cleavage of aggrecan leads to a progressive loss of the G3 domain with aging (9), the hyaluronan-aggrecan complexes are still retained in the tissue. Cross-linking may, however, be of importance in the extracellular matrix formation during development and in response to damage. Support for this notion is found in heart development. In the heart defect (Hdf) mice, truncation of the versican gene by insertion of a transgene resulted in heart malformation and embryonic lethality (24, 25). Interestingly, fibulin-1 and -2 are produced together with versican and hyaluronan in the endocardial cushion tissue (26), suggesting involvement of the fibrins in organization of the hyaluronan-versican complexes of the developing heart extracellular matrix.

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The fibulins are a growing family of extracellular matrix proteins (27). Like fibulin-1, fibulin-2 is an extended protein containing two globular domains. The carboxy-terminal domain III corresponds to the novel FBLC (fibulin/fibrillin carboxy-terminal domain) motif (27). The other globular domain, I, consists of three anaphylatoxin-like repeats. The rod-like domain II, which is composed of EGF-like repeats, connect the two globular domains. Fibulin-2 contains an additional rod-like amino-terminal domain (28, 29). Fibulin-1 can form dimers but is predominantly found as in the monomeric form. Fibulin-2, on the other hand, forms disulfide-linked dimers (30). Interactions between domains II and N give the fibulin-2 dimers a polymorphic shape with X-, Y-, or rod-shaped dimers (30). During development, fibulins are broadly expressed (31–33) with particularly high levels in the developing heart valves (26, 34). In the adult, both fibulin-1 and -2 are found in microfibrils and in many other tissue structures, notably in basement membranes and vessel walls (28, 35, 36).

We here show that fibulin-2 also is a strong ligand for several lectins. They interact through their lectin domains with two different binding sites in domain II of fibulin-2. The dimeric nature of fibulin-2 allows for efficient cross-linking of hyaluronan-lectin complexes, which could play an essential role in the supramolecular organization of cartilaginous and other extracellular matrices.

**Experimental Procedures**

Production of Recombinant Alkaline Phosphatase-tagged Lectin Domains—The construction of His-tagged rCLD mammalian expression pCPE4 plasmids have previously been described (15, 18). A cDNA coding for alkaline phosphatase (AP) was inserted into the pCPE4 plasmid at the SalI site between the lectin domains and the histidine tags. The AP-cDNA was amplified from the AP-tag plasmid (37) using the following primers: 5′-GACCGTGACCATCATTCCGTTGAAGGAAG-3′ and 5′-GACCGTGACCATCATTCCGTTGAAGGAAG-3′. All expression vectors were sequenced before use. The resulting expression vectors code for fusion proteins containing an immunoglobulin signal peptide, a C-type lectin-like domain, soluble alkaline phosphatase, and a hexahistidine tag. The recombinant proteins were produced in human embryonal kidney 293-EBNA cells, purified, and characterized as described previously (18).

Recombinant Fibulin-2 Proteins—The production of most of the recombinant fibulin-2 proteins has been described previously (30). The novel fragments EG + III (position 1036–1195), EG + III (position 993–1195), Iib–e (position 730–912), and Iid–g (position 821–983) were produced by amplifying the corresponding regions of the cDNA. Primers were used in the reverse transcriptase reaction that introduced an Nhel site immediately upstream and an XbaI site immediately downstream the fibulin-2 fragment. The resulting products were then introduced into the pCPE-Pu mammalian expression vector containing the BM-40 signal peptide (38). The recombinant proteins all contain the vector-derived amino acid sequence APLA preceding the first amino acid residue of the fibulin-2 fragment. The recombinant proteins were produced in 293-EBNA cells, purified, and characterized as described previously (30).

Surface Plasmon Resonance Binding Studies—The different rCLDs were diluted with 10 mM sodium acetate, pH 4.0, and immobilized in different flow cells of a CM5 sensorchip (BIAcore, Uppsala, Sweden). Immobilization levels were between 1500 and 2000 resonance units, as described previously (18). For affinity measurements, binding and dissociation were monitored in a BIAcore 2000 instrument. The recombinant proteins were injected at different concentrations over the rCLD-coated surfaces at 35 μl/min (in running buffer: 10 mM Hepes, pH 7.5, 150 mM NaCl, 0.005% surfactant P20, and 1 mM CaCl₂, 25 °C). A flow cell subjected to the coupling reaction without protein was used as a control for bulk resonance changes. The lectin surfaces were regenerated by injection of a 2.0 M pulse of running buffer containing 30 mM EDTA followed by 50 μl of 20 mM CaCl₂ between each experiment. In control experiments with the same concentrations of recombinant proteins, but with 5 mM EDTA instead of CaCl₂ in the running buffer, no binding was seen. Bacterially expressed fibronectin type III repeats 3–5 from rat tenasin-R were used as a positive control (18). After X and Y normalization the blank curves from the control flow cell were subtracted, and association (kₐ) and dissociation (kᵅ) rate constants were determined using a global simultaneous Langmuir fit in the BiaEvaluation 3.0 program. The equilibrium dissociation constants (K_D) were calculated from these values.

Solid Phase Competition Binding Studies—Microtiter wells (NUNC Maxisorp, Naug, Denmark) were coated at room temperature overnight with 1.5 μg/ml tenasin R, fibulin-1C, and fibulin-2 II + III, respectively. The wells were washed with TTBS-Ca (5 mM CaCl₂, 150 mM NaCl, 0.05% Tween 20, 50 mM Tris-HCl, pH 7.4), incubated 2–1 h with blocking solution (3% BSA in TTBS), and washed with BSA/TTBS-Ca (0.5% BSA in TTBS-Ca). Potential inhibitors, i.e., the proteins used for competition were loaded into the wells in triplicates starting at a dilution of 500 nmol together with 1 μg/ml (14 nm) of AP-tagged lectin domain (Ac-AP-his, NcL-AP-his, and VcL-AP-his, respectively) or AP alone in BSA/TTBS-Ca and incubated 1 h. After washing extensively with TTBS-Ca, substrate (1 mg/ml p-nitrophenyl phosphate in 5 mM MgCl₂, 100 mM NaCl, 100 mM Tris-HCl, pH 9.5) was added, and the absorbance at 405 nm was measured after a 1-h incubation.

Extraction of Aggrecan in the Native State—Swarm rat chondrocorosa tumor tissue was dissected free of surrounding fascia and sliced with a razor blade, and 200 g were briefly homogenized (Polytron homogenizer, Kinemartica GmbH, Switzerland) in 1 liter (5 ml/g tissue) of prechilled TTBS-Ca (5 mM CaCl₂, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4) containing 5 mM benzamidine hydrochloride, 5 mM N-ethylmaleimide, and 0.5 mM phenylmethylsulfonyl fluoride. The resulting supernatant was extracted for 45 min at 4 °C with stirring. Fractions, which were collected by centrifugation for 30 min at 20,000 rpm (4 °C, Beckman J2–21, JA-20 rotor, 48, 400 × g). This extraction cycle was repeated twice. The residual tissue was then extracted 3 times, 2 times for 60 min, and finally overnight, as described above in TBS-EDTA (10 mM EDTA) with protease inhibitors. For anoxia exchange a DE52 cellulose (Whatman) 10 × 5-cm column was equilibrated with 20 mM NaCl, 50 mM Tris-HCl, pH 7.4, loaded with supernatants from the Ca⁺² and EDTA extractions, washed, and eluted using a gradient of 20 mM to 2 M NaCl over 2 bed volumes. The flow rate was 25 ml/h, and fractions of 4 ml were collected. Aliquots of the fractions were analyzed for contents of specific proteins by SDS-polyacrylamide gel electrophoresis (PAGE) and sequential Alcian Blue and Coomassie Brilliant Blue R-25 staining. Fractions with proteoglycan retained in the stacking gel were pooled (140 ml) and taken to gel filtration on a Sepharose CL-2B (Amersham Pharmacia Biotech) 100 × 5-cm column equilibrated and eluted with 20 mM NaCl, 50 mM Tris-HCl, pH 7.4. The flow rate was 40 ml/h, and 10-ml fractions were collected. As a final concentration step, size-excluded material of interest was loaded onto another DE52 cellulose (Whatman) 13 × 1-cm column and chromatographed as described above. The flow rate was 10 ml/h, and fractions of 5 ml were collected.

Isolation of Versican from Cell Cultures—MG63 osteosarcoma cells (CRL 1427; American Type Culture Collection, Manassas, VA) were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.). Versican was prepared by anoxia exchange chromatography of conditioned culture medium. For batch binding, 10 ml of Q-Sepharose Fast Flow (Amersham Pharmacia Biotech) was added to 1 liter of medium. Versican was prepared by anion exchange chromatography of conditioned culture medium. For batch binding, 10 ml of Q-Sepharose Fast Flow (Amersham Pharmacia Biotech) was added to 1 liter of medium.

Diet with Chondroitinase ABC—Aggrecan and versican were dialyzed against 100 mM sodium acetate, 100 mM Tris-HCl, pH 7.3. They were digested with 10 milliunits of chondroitinase ABC (Sigma) per mg of proteoglycan for 4 h at 37 °C in the presence of 10 μl of ovomucoid as protease inhibitor. The aggrecan digest was chromatographed on a Superose 6 (Amersham Pharmacia Biotech) 140 × 1-cm column and eluted using a gradient of 0–2 M NaCl over 20 column bed volumes. Versican containing fractions were identified by blotting with monoclonal antibody 12C5 (40), pooled, and concentrated.

Digestion with Chondroitinase ABC—Aggrecan and versican were radiolabeled using 125I and IODOBEADS (Fierce) and were recovered in TTBS-Ca (5 mM CaCl₂, 150 mM NaCl, 0.05% Tween 20, 50 mM Tris-HCl, pH 7.4). Labelled material was affinity-purified on recombinant tenascin-R fibronectin type III repeats 3–5 (15) coupled to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech). After an extensive wash with TTBS-Ca, bound material was eluted with TTBS-EDTA (10 mM EDTA).
Solid Phase Binding Assays with Native Full-length Proteoglycan—Microtiter wells (Breakable Combiplate-Enhanced Binding, Lab-systems Oy, Finland) were coated with 1.5 μg/ml tenascin-R, fibulin-1C, or fibulin-2 in 10 mM sodium carbonate buffer, pH 9.5, overnight at room temperature in a humid chamber. After washing the wells with TTBS (1% BSA, 0.05% Tween 20, TTBS-EDTA, radiolabeled and affinity-purified aggrecan or versican at different concentrations was added at a starting dilution of 100,000 and 40,000 cpm/well, respectively, in triplicates in either TTBS-Ca or TTBS-EDTA. After overnight incubation the wells were extensively washed and counted in a gamma counter (Packard Cobra II Quantum, Packard Instrument Co.).

Electron Microscopy—Glycerol spraying/rotary shadowing, negative staining, and evaluation of the data from electron micrographs were carried out as described previously (41). For rotary shadowing 20-μl samples of complexes between fibulins and native aggrecan or fibulins and rCLDs (typical concentrations 5–10 μg/ml in TBS/5 mM CaCl₂) were adsorbed to 400-mesh carbon-coated copper grids, washed briefly with water, and stained with 0.75% uranyl formate. Since the rCLDs alone were too small to be visualized, we used AP-tagged proteins. After mixing the proteins in equimolar amounts, they were incubated 1 h before adsorption of the formed complexes to grids. As control complexes, affinity-purified, affinity-purified as above, were also mixed with an affinity-purified antibody against the aggrecan lectin domain to distinguish the G3 domain of the core protein. The grids were rendered hydrophilic by glow discharge at low pressure in air. In some experiments the fibulins or rCLDs were labeled with colloidal thiacyanate gold (42). Specimens were observed in a Jeol 1200 EX transmission electron microscope operated at 60-kV accelerating voltage. Images were recorded on Kodak SO-163 plates without preirradiation at a dose of typically 2000 electrons/square nm.

Antibodies and Immunohistochemistry—Mouse tissues were fixed in 25% ethanol, 1% acetic acid, embedded in paraffin, cut into 4-μm sections, and immunostained as described previously (18). Antibodies used were directed against rat aggrecan (18), mouse fibulin-1 (affinity purified) (43), and mouse fibulin-2 (affinity purified) (43). An antiserum could be equally inhibited by all three competitors. Aggrecan rCLD binding to tenascin-R was not inhibited by fibulin-1C in solution, and this rCLD did not bind to a fibulin-1C surface (not shown). Neurocan rCLD and AP alone did not bind to the coated fibulin surfaces (not shown).

Native Full-length Proteoglycans Can Be Affinity-purified on Tenascin-R—To obtain full-length aggrecan, we extracted and

RESULTS

Fibulin-2 Binds Proteoglycan Lectin Domains—Fibulin-2 bound strongly to the rCLDs of aggrecan, versican, and brevican lectin domains in BLAcore experiments (Fig. 1). In contrast, fibulin-2 showed no binding to the neurocan rCLD, whereas this protein was functional and bound tenascin-R (not shown). There was only a very slow dissociation of fibulin-2 bound to aggrecan (Fig. 1A) or versican (Fig. 1C) rCLD, whereas the fibulin-brevican rCLD complex showed more pronounced dissociation (Fig. 1B). All interactions depended completely on calcium ions as expected for C-type lectins (not shown). The \( K_D \) values of the proteoglycan rCLD interactions with fibulin-2 were in the subnanomolar to nanomolar range (Table I).

The Different Proteoglycan Lectin Domain Ligands Compete for the Same Binding Site—In solid phase assays using a surface coated with fibulin-2, we allowed alkaline phosphatase-tagged aggrecan or versican rCLD to bind to the coated protein in the presence of varying concentrations of the different ligands (tenascin-R FnIII-repeat 3–5, fibulin-1C, and fibulin-2). Binding of versican rCLD to the immobilized ligand protein could be inhibited by any of the other proteins (Fig. 2B). Aggrecan rCLD binding to fibulin-2 could only be inhibited by tenascin-R and fibulin-2 but not by fibulin-1C at a 20-fold molar excess (Fig. 2A).

In assays using surfaces coated with the ligands tenascin-R FnIII 3–5 and fibulin-1C (not shown), binding of versican rCLD could be equally inhibited by all three competitors. Aggrecan rCLD binding to tenascin-R was not inhibited by fibulin-1C in

![Figure 1](http://www.jbc.org/)

**FIG. 1.** Fibulin-2 binds proteoglycan C-type lectins. Recombinant full-length fibulin-2 was injected over recombinant proteoglycan C-type lectin domains in a BLAcore 2000. A, aggrecan rCLD. B, brevican rCLD. C, versican rCLD. Injection started at 115 s and ended at 235 s (arrows). No binding was seen to neurocan rCLD (not shown). Bound fibulin-2 was rapidly and completely removed through injection of EDTA (not shown).

| Proteoglycan | Fibulin-2 | Fibulin-1C | Fibulin-1D | Tenascin-R |
|-------------|------------|------------|------------|------------|
| Aggrecan    | 0.1        | 32         | 11         | 12         |
| Versican    | 0.1        | 14         | 38         | 15         |
| Brevican    | 1.8        | NB         | NB         | 1          |
| Neurocan    | NB         | NB         | NB         | 31         |

Data were obtained by surface plasmon resonance analysis. NB indicates no binding.
purified rat chondrosarcoma under native conditions. The associatively extracted aggrecan is present in its ternary complex together with hyaluronan and link protein (44). After digestion with chondroitinase ABC, the core protein was radioiodinated and affinity-purified on the rCLD-binding tenascin-R FnIII 3–5 fragment (Fig. 3A). Bound aggrecan was eluted with EDTA, taking advantage of the calcium dependence of CLD binding. SDS-PAGE analysis of the eluted material (Fig. 3B) shows accumulation of aggrecan core protein in the full-length molecular weight range, fragments, and copurified link protein. We confirmed that the eluted material consisted of full-length aggrecan core protein rich in globular domains G1, G2, and G3 (9) by glycerol spraying/rotary shadowing electron microscopy (Fig. 3D). The nonbinding fraction, however, contained the pair of G1 and G2 domains at the amino terminus but lacked in most cases the carboxyl-terminal domain G3 (Fig. 3C).

Fibulin-2 Cross-links Aggregating Proteoglycans

Affinity-purified Proteoglycans Bind Lectin Domain Ligand in Solid Phase Assays—In the proteoglycan G3 domain the CLD is flanked by EGF and sushi repeats. To determine whether these motifs influence the CLD ligand binding, we performed solid phase binding assays with the affinity-purified radiiodinated full-length aggrecan. The full-length proteoglycan indeed shows the same binding characteristics as the aggrecan rCLD. Aggrecan binds tenasin-R (Fig. 4A) and fibulin-2 (Fig. 4C) in a calcium-dependent manner, whereas no
binding was observed to fibulin-1C (Fig. 4B). Full-length versican isolated from cell cultures was radiolabeled and affinity-purified using the same protocol as for aggrecan (not shown). In solid phase assays, versican bound tenascin-R (Fig. 4D), fibulin-1 (Fig. 4E), and fibulin-2 (Fig. 4F), which was dependent on calcium and comparable to the versican rCLD.

The Proteoglycan rCLD Interacts with the Calcium Binding EGF Repeats of Fibulin-2—The interaction sites for the proteoglycan rCLDs on fibulin-2 were mapped using a collection of different fragments of the molecule (Fig. 5A) recombinantly expressed in 293-EBNA cells. As displayed in Table II, the central domain II (fragment II) is sufficient for proteoglycan rCLD binding with similar affinities as for the full-length fibulin molecule. Binding, albeit weak, was also observed to some subfragments of domain II (fragments EG1III, (EG)2III, and IIb–e) but not to fragment IId–g. This suggests the presence of two cooperative binding sites for the proteoglycan rCLDs on the fibulin-2 molecule, as outlined in Fig. 5B. The previously mapped binding sites on fibulin-1C (18) are shown for comparison.

Electron Microscopy—We used negative staining electron microscopy to confirm the results of the mapping experiments. Fibulin-1C was found to be dumbbell-shaped monomers (Fig. 6A) as previously reported using rotary shadowing microscopy (45). Fibulin-2 (Fig. 6B) was found predominantly as X-shaped dimers (compare schematic in Fig. 5A, top). A morphometric analysis was based on a large set of molecular complexes (Fig. 6, G–N). In agreement with the different numbers of EGF-like repeats present in the two fibulins, the center-to-center distances between the globular domains in fibulin-1C and fibulin-2 were found to be 15 and 20 nm, respectively (Fig. 6, G and H). In the latter case the two globular domains I and III could be separately identified due to the additional N-domain. Gold-labeled aggrecan rCLD (Fig. 6, C and D) and versican rCLD (Fig. 6, E and F) bound to the central stretch of calcium binding EGF-like repeats in domain II of both fibulin-1C (Fig. 6, C and E) and fibulin-2 (Fig. 6, D and F). When we measured the distance from the bound proteoglycan lectin domain to the closest globular domain of fibulin-1C, we found that the aggrecan binding distribution was restricted to a narrow region of domain II close to that globular domain (Fig. 6I). The versican binding was more widely distributed along domain II (Fig. 6M). These interaction sites correspond well with the BIAcore bind-
ing site mapping (Fig. 5B). Aggrecan rCLD binding to fibulin-2 showed a bimodal distribution on domain II with some binding close to the globular domain I (Fig. 6J) and more extensive binding closer to domain III, also in correspondence with biochemical data (Fig. 5B). Versican rCLD showed a more equal binding distribution for the same two regions along domain II (Fig. 6N), comparable to mapping data. More than 70% of the aggrecan and versican rCLDs were bound, whereas brevican and neurocan rCLDs showed no significant binding (below 5% of total rCLD) to both fibulins. Interactions of unlabeled native full-length aggrecan with fibulin-1 (Fig. 6K) and fibulin-2 (Fig. 6L) were shown to be in accordance with those of the lectin domain alone. Interestingly, both negative staining (Fig. 7) and rotary shadowing (not shown) electron microscopy data of full-length aggrecan and fibulins-1 and -2 demonstrated the ability of dimers of both fibulins to act as cross-linkers between different hyaluronan-aggrecan complexes. The binding was to the aggrecan G3 domain positively identified by antibodies against the C-type lectin. There was no indication of an association of the fibulins with the filament representing hyaluronan with

### Table II

Mapping of the binding site on fibulin-2 for the proteoglycan C-type lectin-like domains by surface plasmon resonance affinity measurements

The units used are as follows: $k_a$, M$^{-1}$ s$^{-1}$ $\times 10^{-3}$; $k_d$, s$^{-1}$ $\times 10^{6}$; $K_D$, nM.

| Ligand | Aggrecan | Brevican | Versican |
|--------|----------|----------|----------|
|        | $k_a$    | $k_d$    | $K_D$    | $k_a$    | $k_d$    | $K_D$    | $k_a$    | $k_d$    | $K_D$    |
| Na     | 259      | 18.3     | 0.1      | 287      | 523      | 1.8      | 330      | 36.8     | 0.1      |
| N      | NB*      | NB       | NB       | NB       | NB       | NB       | NB       | NB       | NB       |
| N + 1  | NB       | NB       | NB       | NB       | NB       | NB       | NB       | NB       | NB       |
| I      | NB       | NB       | NB       | NB       | NB       | NB       | NB       | NB       | NB       |
| II + III | 317   | 252      | 0.8      | 310      | 3370     | 10.9     | 280      | 8.9      | 0.03     |
| II     | 735      | 487      | 0.7      | 228      | 11,400   | 50.2     | 354      | 134      | 0.4      |
| EG + III | 26.4  | 5350     | 203      | 17.6     | 12,800   | 727      | 15.4     | 14,400   | 873      |
| (EG)$_2$ + III | 10.7 | 2050     | 186      | 12.8     | 7140     | 559      | 11.4     | 6810     | 394      |

*NB indicates no binding.
and 20 nm in A and C, respectively. The aggrecan G3 domains are denoted by arrowheads. The aggrecan lectin domain antibodies (arrows) as visualized using negative staining electron microscopy. The black dots in the panels are colloidal thioxyanate gold attached at the globular domains of the fibulin molecules. Both panels are sections of larger networks. The aggrecan interactions with fibulin-1 and -2 are shown in detail in E and F, respectively. The aggrecan G3 domains are denoted by arrowheads. In E and F the G3 domain (arrowheads) is identified by the use of anti-aggrecan lectin domain antibodies (arrows). Scale bar = 80 nm in A and B and 20 nm in C–F.

DISCUSSION

In this work we demonstrate that fibulin-2 is a high affinity ligand for the C-type lectin domains of the proteoglycans aggrecan, brevican, and versican. The affinities for versican and aggrecan rCLDs are in the sub-nanomolar to nanomolar range, as measured by surface plasmon resonance technology. No binding to the neurocan rCLD could be detected. Furthermore, we show that fibulin-2 competes for the same binding site as tenasin-R on the aggrecan and versican rCLDs and at least on the versican rCLD also with the site for fibulin-1 binding. In addition, we confirm that full-length proteoglycans can interact with the rCLD ligands by affinity chromatography on tenasin-R and in solid phase assays bind to tenasin-R and fibulin-2. We do not yet have an explanation why no interaction is seen between fibulin-1 and aggrecan rCLD or full-length aggrecan in the solid phase assay. Adsorption may, however, lead to conformational changes in the fibulin-1 molecule affecting binding site functionality. In previous work, we did find a strong calcium-dependent interaction between the aggrecan rCLD and fibulin-1 in solution through copurification and through surface plasmon resonance assays (18). However, in contrast to the versican rCLD interaction, fibulin-1 does not compete for aggrecan rCLD binding to tenasin-R or fibulin-2 in solid phase assays. This indicates separate binding sites for tenasin-R/fibulin-2 and fibulin-1 on the aggrecan lectin domain, but this awaits more detailed analysis. In this respect it is interesting that the versican lectin domain showed good binding. This may indicate differences in the binding sites of the lectins. In agreement with BIAcore data, in solid phase assays neurocan rCLD linked to AP showed no binding to the fibulins nor did AP alone.

Mapping of the interaction site on the fibulin-2 molecule revealed that the proteoglycan rCLDs bind to two nonoverlapping sites on calcium binding EGF-like repeats in the central rod-like domain of the molecule. We observed a large drop in $K_D$ for the interaction with lectic rCLDs when using shorter fibulin-2 fragments containing only one binding site. This probably reflects cooperative binding of the two sites in the larger fragment to the multiple rCLD molecules immobilized on the BIAcore flow cell surface. Measurements of the distance between the gold-labeled rCLDs and the globular domains of the fibulins in negative staining electron microscopy showed distinct narrow binding of aggrecan rCLD and full-length core protein to the parts of domain II in fibulin-2 corresponding to the binding sites observed in the BIAcore mapping experiments. In the fibulin-1 case we also found a narrow binding site for the aggrecan rCLD and core protein, which presumably represents the carboxyl-terminal site of interaction disclosed in the BIAcore experiments. The versican rCLD showed a wider range of binding within domain II of fibulin-1 and two distinct populations of binding sites for fibulin-2. This is in good agreement with the BIAcore mapping of the fibulin binding sites. By using negative staining electron microscopy, we were also able to observe directly fibulin-mediated cross-linking of hyaluronan-aggregan complexes. The fibulin-2 dimers were under these experimental conditions predominantly found in the X-shaped form (30). We failed to observe cross-linking mediated by the rod- or Y-shaped fibulin-2 dimers, suggesting that the interactions between domains N and II may render the lectic-binding sites inaccessible. Fibulin-1 was mostly found in monomeric form although some dimers were observed. Since only dimers could act as aggrecan cross-linkers, fibulin-1 may be less efficient in organizing hyaluronan-lectican complexes.

Immunohistochemical staining show that both fibulins are codistributed with proteoglycan in different tissues, suggesting that the examined interactions are of physiological relevance. Striking examples are the strong expression of versican and both fibulins in the endocardial cushion tissue during heart development (26, 31, 32, 34, 46) and the prominent expression of aggrecan and both fibulins in newly formed cartilage and bone. Indeed, treatment of endocardial cushion tissue sections with hyaluronidase to degrade hyaluronic acid led to removal of both versican and fibulins, whereas fibronectin staining remains unaffected (26). Furthermore, disruption of the versican gene results in heart malformation and early embryonic death (24, 25). This indicates that the correct formation of a hyaluronan-versican-fibulin network may be important for heart development.

Both fibulins are present in precartilaginous mesenchymal condensations and developing cartilage (18, 32, 33). It may be that fibulin-1 and -2 binding by the aggrecan C-type lectin domain plays a significant role during development of cartilage and bone, perhaps in the organization process of the early matrix. Indeed, in the embryonic day 15.5 femur growth plate aggrecan and both fibulins are expressed in an overlapp-
Fibulin-2 Cross-links Aggregating Proteoglycans

FIG. 8. Immunohistochemical staining for fibulin-1, fibulin-2, and aggrecan. Fetal femur growth plate embryonic day 15.5, adult articular cartilage, and adult ear cartilage were paraffin-embedded and cut in 4-μm thick sections. These were stained with antibodies against fibulin-1 (left panels), fibulin-2 (center panels), or aggrecan (right panels). Scale bar = 100 μm.

The proteoglycan rCLDs bind to fibulin-1 and -2 through EGF-like repeats but bind to a fibronectin type III repeat in tenasin-R (15) and tenasin-C (16). Interestingly, the lectin binding is in all these cases directed to extended rod-like stretches of multimeric extracellular matrix molecules. Although the biological functions of these interactions remain to be investigated, the notion that all four ligands form multimeric complexes in the extracellular matrix suggests that these proteins act to cross-link the proteoglycan-hyaluronan complexes in the tissue.

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