Biglycan Organizes Collagen VI into Hexagonal-like Networks Resembling Tissue Structures*

Received for publication, July 10, 2002, and in revised form, September 25, 2002
Published, JBC Papers in Press, September 26, 2002, DOI 10.1074/jbc.M206891200

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The ability of the leucine-rich repeat (LRR) proteins biglycan, decorin, and chondroadherin to interact with collagen VI and influence its assembly to supramolecular structures was studied by electron microscopy and surface plasmon resonance measurements in the BIAcore 2000 system. Biglycan showed a unique ability to organize collagen VI into extensive hexagonal-like networks over a time period of only a few minutes. Only the intact molecule, substituted with two dermatan sulfate chains, had this capacity. Intact decorin, with one dermatan sulfate chain only, was considerably less efficient, and aggregates of organized collagen VI were found only after several hours. Chondroadherin without glycosaminoglycan substitutions did not induce any ordered collagen VI organization. However, all three related LRR proteins were shown to interact with collagen VI using electron microscopy and surface plasmon resonance. Biglycan and decorin were exclusively found close to the N-terminal parts of the collagen VI tetramers, whereas chondroadherin was shown to bind close to both the N- and C-terminal parts of collagen VI. In the formed hexagonal networks, biglycan was localized to the intra-network junctions of the collagen VI filaments. This was demonstrated by electron microscopy after negative staining of gold-labeled biglycan in aggregation experiments with collagen VI.

Collagen VI is unique among the collagens in its molecular and fibrillar arrangement. The monomer consists of three genetically distinct peptide chains (α1(VI), α2(VI), and α3(VI)) that form a triple helical, central domain, flanked by two globular domains at the N and C termini. The N-terminal globular domain is larger than the C-terminal domain and consists almost exclusively of the α3(VI) chain, which has nearly twice the mass of the α1(VI) and α2(VI) chains (1, 2). Collagen VI monomers assemble intracellularly into disulfide-bonded tetramers that are secreted into the extracellular matrix (3). They assemble in a characteristic end-to-end fashion into thin (3–10 nm) beaded filaments as well as hexagonal networks (4–6).

Collagen VI has been identified in microfibrillar assemblies in the extracellular matrix of connective tissues as well as in netlike structures, broad banded “zebra” fibrils with a periodicity of 100 nm and as hexagonal networks (4, 7). When collagen VI is isolated by pepsin digestion, most of the N- and C-terminal domains are cleaved off, leaving the triple helical region in minor parts of the globular domains of the collagen VI molecules, which is still sufficient for forming the tetramer (6, 8).

Collagen VI is present in most tissues. It is enriched close to cells and around basement membranes. It is associated with structures like blood vessels and nerves and found interspersed among interstitial collagen fibers (for reviews see Refs. 2 and 9). In Bethlem myopathy, a dominantly inherited disorder characterized by muscle weakness and wasting, decreased presence of secreted collagen VI due to mutations in COL6A1 has been reported (10, 11). Mutations of a single glycine in the N2 domain of the α3(VI) chain that are likely to lead to misfolding and subsequent degradation of this domain have been identified in another family with Bethlem myopathy (12). Collagen VI deficiency in mouse induces a myopathy with similar characteristics (13). The expression of collagen VI is up-regulated in osteoarthritis (14) and during wound healing (15), suggesting a role for this collagen in tissue integrity and remodeling.

Collagen VI interacts with a broad range of molecules in vitro. Examples are cell surface receptors like the α1β1 and α2β1 integrins, basement membrane components like collagen IV and perlecain, as well as extracellular matrix constituents like the fibrillar type I and II collagens (16–20). Altogether, the complexity of the collagen VI interactions suggests a possible role as a bridging molecule in tissues. Collagen VI also interacts with proteoglycans, including those like the cell surface proteoglycan NG2 (21) and the small leucine-rich repeat (LRR)¹-proteoglycans biglycan and decorin (19, 22). These members of the LRR protein family consist of core proteins containing 10 LRRs substituted with two (biglycan) or one (decorin) dermatan/chondroitin sulfate chains, respectively (reviewed in Refs. 23 and 24). Chondroadherin (25–27) is a leucine-rich repeat connective tissue protein that lacks glycosaminoglycan substituents.

The small proteoglycans decorin, fibromodulin, and lumican are known to interact with collagens I and II and influence their fibril formation in vitro (28–31) and appear to control the fibril diameter in vivo (32–34). Thus, the small LRR-proteoglycans have important roles in controlling the extracellular matrix modeling and remodeling in connective tissues.

In this investigation we addressed the role of selected LRR proteins/proteoglycans in the control of collagen VI network

* This work was supported by the Swedish Medical Research Council, Greta and Johan Kock’s Foundation, the Swedish Rheumatism Association, Alfred Osterlund’s Foundation, Konung Gustaf V’s 80-årsfond, and the Medical Faculty, Lund University. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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This paper is available on line at http://www.jbc.org
formation in vitro. We show a novel role for biglycan in supramolecular organization of collagen VI into hexagonal-like networks resembling those found in some tissues.

EXPERIMENTAL PROCEDURES

Collagen VI—Collagen VI was prepared from placenta after pepsin digestion (35). In short, human placenta was homogenized in formic acid and incubated with pepsin for 24 h at room temperature. After a series of salt precipitations, the collagen VI was purified on an agarose A5m column, dialyzed into dilute acetic acid, and freeze-dried.

Intact collagen VI was purified from bovine cornea (36). In short, bovine corneas were cut into pieces and homogenized in Tris/saline buffer to depolymerize the collagen VI microfibrils. Collagen VI tethers were separated from larger aggregates by a second gel filtration on the Superose 6 column as described.

Recombinant LRR Proteins—Human biglycan (37) cDNA and bovine decorin (38) cDNA were ligated into the Epstein-Barr virus-based eukaryotic expression vector pCEP4 (In Vitrogen, San Diego, CA) and expressed in human HeLa cells and Chinese hamster ovary cells, respectively (39). Cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. Medium was collected, and small proteoglycans were purified by ion exchange chromatography and purified from traces of bovine serum albumin on Blue Sepharose CL-6B (Amersham Biosciences) as described previously (22).

Recombinant chondroadherin was expressed in human kidney cells constitutively expressing EBNA-1 protein from Epstein-Barr virus (293 EBNA). Collected medium from the cultured cells was applied onto a CM-52 column, and proteins were eluted with a sodium chloride gradient (40).

Dermatan Sulfate—Biglycan from bovine articular cartilage (prepared as described earlier (41, 42)) was dissolved in 50 mM ammonium bicarbonate, pH 7.8, at a concentration of 1 mg/ml protein, reduced with 5 mM 1,4-dithiothreitol (Merck, Darmstadt, Germany) for 30 min at room temperature and alkylated with 20 mM iodoacetamide (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) for 90 min in the dark at room temperature. The sample was diluted into 2 mM guanidine hydrochloride and digested with sequencing grade modified trypsin (Promega, Madison, WI) for 24 h at 37 °C. The digest was dialyzed against 20 mM Tris, pH 7.4, and applied onto a 5-ml DEAE-Sepharose Fast Flow column (Amersham Biosciences, Upsala, Sweden) equilibrated in the same buffer. The peptide fragment carrying two glycosaminoglycan chains was eluted with a linear gradient of 0–1 M sodium chloride in 20 mM Tris, pH 7.4, dialyzed against water, and frozen.

Chondroitinase ABC Digestion—Biglycan was diluted in TBS to a final concentration of 400 nM, and 50 milliunits/ml chondroitinase ABC (Seikagaku Corp., Tokyo, Japan) was added. Samples were incubated at 37 °C for 0.5–2 h with the progress of the digestion monitored at 232 nm. Digestion was further checked by the appearance of core protein visualized by SDS-PAGE.

Alternatively, digestion with chondroitinase ABC of pre-formed collagen VI/biglycan networks (described below) were done overnight at room temperature with chondroitinase ABC at the same enzyme to substrate ratio as described above.

Interactions Studied by Surface Plasmon Resonance in the BIAcore2000 System—Pepsin extracted collagen VI (200 μg/ml in 10 mM sodium citrate, pH 3.2) was covalently immobilized on the BIAcore Sensorchip CM-5 (carboxylated dextran matrix) with a 1:1 mixture of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and N-hydroxysuccinimide (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) according to the description of the manufacturer. Remaining active groups on the matrix were blocked with 1 M ethanolamine-HCl (BDH Chemicals, Poole, UK) at pH 8.5. The running buffer was 10 mM HEPES, pH 7.4, 150 mM sodium chloride, 3.4 mM EDTA, 0.005% (v/v) surfactant P20 (BIAcore AB, Upsala, Sweden).

Studies of interactions were performed in TBS, pH 7.4, containing 0.05% surfactant P20. LRR proteins/proteoglycans (100–200 nM) were injected over immobilized collagen VI at a flow rate of 20 μl/min. Individual experiments were performed between five and ten times.

Interaction of Gold-labeled LRR Proteins with Collagen VI Tetramers—Colloidal gold particles of 4 nm ± 15% were prepared by reduction of HAuCl4 by thiocyanate (43) and, after titration, conjugated to purified recombinant biglycan, decorin, and chondroadherin as described previously (44). Gold-labeled LRR proteins/proteoglycans (at 20 nM) were mixed with intact collagen VI from bovine cornea in TBS, and the mixture was instantly adsorbed onto a 400-nm carbon-coated copper grid, which was rendered hydrophilic by glow discharge at low pressure in air. The grid was immediately blotted, briefly washed with 2 drops of water, and stained with 0.75% uranyl formate for 15 s. Specimens were studied in a Jeol 1200 EX transmission electron microscope operated at 60-kV accelerating voltage and ×75,000 magnification.

Studies of Collagen VI Network Formation—Small LRR proteins/proteoglycans (at 20–30 nM) or a trypsin fragment of biglycan containing a short peptide with the two glycosaminoglycan substituents (at 10–80 nM) were incubated at room temperature for 5 min and 18 h, respectively, with collagen VI (at 20 nM) in TBS. Samples were immediately taken to negative staining for electron microscopy as described above. For localization of biglycan in complexes with collagen VI, col-
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RESULTS

Interactions Studied by Plasmon Resonance—Immobilization of collagen VI resulted in surface concentrations of about 6 ng/mm² (6000 resonance units). The three LRR proteins studied, biglycan, decorin, and chondroadherin, all interacted with collagen VI (Fig. 1, upper panel).

Interactions of Gold-labeled LRR Proteins/Proteoglycans with Intact Collagen VI—Complexes were formed within a few minutes after mixing individual components and visualized by electron microscopy after negative staining. Five to ten experiments were carried out for each condition, and some 300 complex particles were evaluated. Whereas biglycan (left) and decorin (middle) bound only close to the N-terminal parts of collagen VI, chondroadherin (right) was found both close to the globules corresponding the N-terminal and the C-terminal parts of the collagen VI molecules in the tetramers (Fig. 1, lower panel). Over 85% of the complexes exhibited only one gold-labeled decorin or biglycan molecule per collagen VI N-terminal or only one gold-labeled chondroadherin particle per collagen VI N- and C-terminal, respectively. These numbers did not vary significantly between the experiments. No binding to the filamentous triple helical portion of the collagen was observed.

Collagen VI Network Formation in the Presence of LRR Proteoglycans—Collagen VI, incubated with recombinant biglycan at room temperature and then visualized with electron microscopy after negative staining, formed networks extending over...
several micrometers already after a few minutes (Fig. 2).
There was no difference between intact collagen VI (Fig. 2a) and collagen VI that had been digested with pepsin, which does not affect biglycan binding to the collagen (Fig. 2b). Upon magnification, these networks exhibited an organized, hexagonal type of structure in all cases (Fig. 3). Further studies were performed with pepsin-extracted collagen VI for practical reasons. Between 300 and 500 different networks were evaluated in these and the following experiments.

By labeling biglycan with 3.5-nm colloidal gold before incubation with collagen VI, it became possible to identify its localization to the intra-network junctions that appeared with a distance of about 100 nm (Fig. 3a). The incorporation of biglycan reached its saturation at concentrations around 5 nM when the ratio of biglycan:collagen VI was 1:4 (Fig. 3b). The dependence of collagen VI network formation on biglycan concentration was investigated. At a molar ratio of 3:4 (biglycan:collagen VI), the mean surface area of the collagen VI networks was not significantly changed upon further addition of biglycan (Fig. 4a). To permit tracing of biglycan in the collagen networks, the proteoglycan was labeled with gold. In control experiments it was shown that this labeling did not alter the effects on the collagen network formation (Fig. 4b).

The organization of the collagen VI networks formed was further studied. In the presence of biglycan they did not appear to change compared with 5 min when the incubation time was extended overnight (Fig. 5, Bgn). When collagen VI was incubated with recombinant decorin at the same molar ratio to collagen as that used for biglycan, the formation of organized networks was much slower (Figs. 5 and 6). Although at early times the network formation was poor, after very extended incubation times they resembled those obtained with biglycan present (Fig. 5, Dcn). Biglycan and decorin extracted and purified from tissue under denaturing conditions showed the same effect as the recombinant proteoglycans (not shown). To further investigate the different effects of the two proteoglycans, we chose to focus on the role of the glycosaminoglycan substituents for their ability to assist in organizing collagen VI. Biglycan core protein, in which glycosaminoglycan chains had been removed by chondroitinase ABC digestion prior to incubation with collagen VI, did not induce collagen VI network formation. Thus, only small clusters (up to 500 nm²) of collagen VI without any apparent organization were found after as much as 18 h of incubation (Fig. 5, Bgn-core). These small
clusters showed no difference from those formed when collagen VI was incubated alone under the same conditions (Fig. 5, CHAD). Chondroadherin, which lacks glycosaminoglycan chains while being able to bind close to the globular domains of collagen VI (Fig. 1, CHAD), was consistently unable to catalyze network formation (Fig. 5, CHAD). Incubation of collagen VI with the trypsin-released peptide containing the two glycosaminoglycan chains did not catalyze network formation (not shown). The networks formed in the presence of intact biglycan did not appear different when isolated dermatan sulfate peptides or biglycan core protein were added as putative competitors (data not shown). Addition of dermatan sulfate or biglycan core protein to the preformed collagen VI-biglycan networks did not disrupt formed networks nor did digestion with chondroitinase ABC (data not shown).

A closer examination of the kinetics of the formation of collagen VI hexagonal networks under the influence of intact biglycan as well as decorin was performed with incubations for different times at room temperature. The mean areas of the networks formed were measured and plotted against time. We found a significant difference in the ability of biglycan compared with decorin to catalyze the formation of hexagonal collagen VI networks. When intact biglycan is present, networks with an average surface area of 40 μm² are formed within less than 5 min. After 5 min, further modification of the networks is slow. In the presence of decorin, there is very limited network formation even after several hours. However, at 24 h of incubation networks formed in the presence of decorin resemble those in the presence of biglycan (Fig. 6).

Even if the formation of collagen VI networks with biglycan was a fast process, smaller aggregates could be captured and visualized at time = 0. Here we found structures that appeared to form different pre-stages of hexagonal networks where laterally aligned collagen VI tetramers gradually were replaced by larger clusters organized as hexagonal stars (Fig. 7).

**DISCUSSION**

Biglycan as well as decorin have previously been shown to interact with the N-terminal part of pepsin-treated collagen VI (22). This interaction was not dependent on the glycosaminoglycan side chains. In the present study we show a novel role for biglycan in collagen VI network formation and clearly demonstrate that also the glycosaminoglycan side chains have an important function.

Biglycan, which is the only LRR protein substituted with two glycosaminoglycan chains, showed a unique ability to organize collagen VI into a hexagonal-like network in a very short period of time, i.e. only a few minutes. This appears to represent a biologically relevant property, because collagen VI networks with similar appearance have previously been found in tissue (4). Decorin substituted with only one glycosaminoglycan chain also catalyzed the formation of collagen VI networks but was much less efficient, requiring a distinctively longer time to do so. In cartilage, where collagen VI is enriched in the pericellular capsule of chondrons (45), biglycan is predominantly located
in the pericellular matrix while decorin is located interterritorially (46, 47). High resolution co-localization studies of collagen VI and the LRR proteins in different tissues will provide important information of their roles in vivo. Although our results strongly suggest that biglycan has a role in collagen VI network formation and function, the presence of such interactions in vivo remains to be established. Also the different effects of decorin and biglycan on collagen VI assembly may be relevant to the fine-tuning of the process. The roles of the proteins may differ between tissues.

The presence of apparent pre-stages of collagen VI networks in the presence of biglycan allows us to establish a model that is likely to be representative for the network formation (Fig. 7). This appears to occur via adherence of the outer, N-terminal parts of collagen VI to each other. These bundles further associate via their outer ends into an organization of whole “stars” that gradually assemble into larger aggregates.

The localization of gold-labeled biglycan to the intra-network junctions, corresponding to the N-terminal part of the collagen VI molecules, fits well with our previous results (22). Biglycan digested with chondroitinase ABC to remove the glycosaminoglycan chains as well as chondroadherin lacking such chains showed no ability to organize collagen VI, demonstrating that the glycosaminoglycans play a crucial role in the process of collagen VI network formation. Interestingly, fibromodulin, with one or two keratan sulfate chains different from those dermatan sulfate chains in biglycan and decorin, shows the same effect as decorin (not shown), thus indicating that the specific properties of the glycosaminoglycan substitution might be less important than the clustered, negatively charged groups.

In a recent study (48) it was shown that the secondary structure of biglycan and decorin was not altered by digestion with chondroitinase ABC. This structure, however, appeared to be influenced by its post-translational modifications with oligosaccharides. It is thus possible to add an additional independent functional domain via glycosaminoglycan substitution as well as chondroadherin lacking such chains, shows the same effect as decorin (not shown), thus indicating that the specific properties of the glycosaminoglycan substitution might be less important than the clustered, negatively charged groups.

Recently mice deficient in one or two of the four most prominent and widely expressed LRR proteoglycans (biglycan, decorin, fibromodulin, and lumican) were generated (for review see Ref. 51). The deficiencies in decorin or biglycan resulted in abnormal collagen I and II fibril architecture. Deficiency in both proteoglycans enhanced this effect. Thus the collagen phenotypes demonstrated a cooperative, sequential, timely or orchestrated action of these molecules that shape the architecture and mechanical properties of the collagen matrix. The data now presented show that it will be highly relevant to extend our studies on the collagen VI matrix in tissues, which lack LRR proteoglycan gene products.

Together with previous results (22), it is now becoming apparent that biglycan and decorin interact with collagen VI via their core proteins at the same time as their glycosaminoglycan chains appear to have a crucial role in guiding collagen VI into the organized structures observed in this study as well as in tissue (4). This conclusion is supported by the fact that other members of the LRR protein family not substituted with glycosaminoglycan chain(s), albeit able to bind to collagen VI, do not induce the formation of extended organized networks. Neither the core protein nor isolated glycosaminoglycan chains nor the isolated peptide with the double glycosaminoglycan chains inhibits the process. This indicates that an important cooperative binding of the two sites occurs in the soluble phase that is apparently not seen in, for example, surface plasmon resonance. These apparent contradictory results can be explained if the proteoglycans bind via their core protein and their glycosaminoglycan chains subsequently serve to keep the collagen molecules separated while assembly is initiated.

Acknowledgments—We appreciate the skillful technical assistance of Maria Baumgarten. The help of Rita Wallen and Eric Hallberg from the Institute for Cell and Organism Biology is gratefully acknowledged.

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