Native supramolecular assemblies containing collagen VI microfibrils and associated extracellular matrix proteins were isolated from Swarm rat chondrosarcoma tissue. Their composition and spatial organization were characterized by electron microscopy and immunological detection of molecular constituents. The small leucine-rich repeat (LRR) proteoglycans biglycan and decorin were bound to the N-terminal region of collagen VI. Chondroadherin, another member of the LRR family, was identified both at the N and C termini of collagen VI. Matrilin-1, -3, and -4 were found in complexes with biglycan or decorin at the N terminus. The interactions between collagen VI, biglycan, decorin, and matrilin-1 were studied in detail and revealed a biglycan/matrilin-1 or decorin/matrilin-1 complex acting as a linkage between collagen VI microfibrils and aggrecan or alternatively collagen II. The complexes between matrilin-1 and biglycan or decorin were also reconstituted in vitro. Colocalization of collagen VI and the different ligands in the pericellular matrix of cultured chondrosarcoma cells supported the physiological relevance of the observed interactions in matrix assembly.

Connective tissues are characterized by an abundant extracellular matrix in which a wide variety of different proteins and proteoglycans assemble into multimolecular complexes, often in the form of networks. The fibrillar collagens are major components (for review, see Ref. 1) and, in cartilage, collagen II forms cross-striated fibrils in association with collagen IX and XI (2). Collagen VI is another member of the collagen family that distinguishes itself by containing large globular domains at its N and C termini (3–6). The molecule consists of three genetically distinct o-chains, α1(VI), α2(VI), and α3(VI). The N-terminal globular region is composed of nine or ten von Willebrand factor (vWF) A-like domains derived from the α3-chain. Collagen VI molecules associate laterally in an antiparallel fashion into dimers that are stabilized by disulfide bridges (3, 4, 7). The dimers aggregate further into tetramers that are secreted into the extracellular matrix (7), where they join end to end into microfibrils. These subsequently form characteristic thin beaded filaments that are found in a variety of tissues (3, 8, 9). The formation of microfibrils was recently shown to depend on the N5 vWFA-like domain of α3(VI) (10).

In addition to the collagens, the large hyaluronan-binding proteoglycan, aggrecan is a major constituent of the cartilage extracellular matrix. The aggrecan core protein has a molecular weight of about 220 kDa (11) and is heavily substituted with about 100 chondroitin sulfate chains, 30 keratan sulfate chains, and 60 N- and O-linked oligosaccharides (12–16). Aggrecan interacts with hyaluronan and other matrix molecules (reviewed in Ref. 17). These major constituents provide the basic organization of the extracellular matrix, while other molecules modulate its assembly and structure. The matrilins are a family of oligomeric matrix proteins containing common structural motifs such as vWFA-like domains, epidermal growth factor-like EGF modules and coiled-coil regions (reviewed in Ref. 18). Matrilin-1 (also known as cartilage matrix protein, CMP), and matrilin-3 are abundant in cartilage (19, 20, 21) while matrilin-2 and matrilin-4 show a broader tissue distribution (22–25). All matrilins form homo-oligomers through assembly of coiled-coil structures from a common C-terminal stretch of heptad repeats. In addition, matrilin-1 and -3 form hetero-oligomers in bovine epiphyseal cartilage (21, 26). Matrilin-1 has been shown to interact with fibrillar collagens (27) and aggrecan (28, 29).

The LRR (leucine-rich repeat) protein family consists of 12 known extracellular members and the molecules appear to play major roles in modulating the functional properties of the collagen networks (17, 30). Nine of the members are characterized by a core protein with 10 to 11 leucine-rich repeats, surrounded by disulfide-linked loops. Most LRR proteins have an N-terminal extension with specific characteristics. These range from clustered tyrosine sulfates, long stretches of aspartate residues, clustered basic amino acids as well as substitution with oligosaccharides or glycosaminoglycan chains. In many cases binding to triple helical collagens has been demonstrated. Decorin, a proteoglycan member of this family, interacts with fibrillar collagens in vitro (31) and interferes with collagen fibrillogenesis (32, 33). It has also been shown to interact with collagen VI (34) at the same site as biglycan (35), another...
LRR-proteoglycan closely related to decorin. Biglycan is substi-
tuted with two glycosaminoglycan chains, often dermatan
sulfate, in its N-terminal domain, while decorin carries a single
chain (36–40). Biglycan does not appear to be involved in the
assembly of the fibrillar collagens. However, it was recently
shown to bind to the vicinity of the collagen VI N-terminal
by its core protein in a glycosaminoglycan-independent manner
(32). Furthermore, biglycan was demonstrated to induce and
catalyze the formation of hexagonal-type collagen VI networks
in vitro, a function that depends on its glycosaminoglycan
substitution (41).

The tissue-specific structures and functions of the extrace-

cellular matrix involve complex, and as yet poorly understood,
interactions between individual extracellular proteins. The for-
mation of these specific architectural elements is critical for
normal tissue development and function. The present study
represents a detailed examination of networks of molecules
isolated under mild conditions from the Swarm rat chondrosar-
coma. We use molecular electron microscopy in combination
with immunogold techniques to show that small LRR-proteo-
glycans together with matrilins form a linkage between colla-
gen VI microfibrils and other macromolecular components
in the cartilage extracellular matrix.

EXPERIMENTAL PROCEDURES

Preparation of Chondrons—Chondrons were isolated from rat
chondrosarcoma tissue by a modification of a previously published method
(42). In short, freshly isolated tumor tissue was dispersed in Nutrient
Mixture F-12 (HAM’s F 12) (Invitrogen) by passing it through a nylon
filter (pore size 50 μm). The dispersed tissues were washed with
HAM’s F12 on a 25 μm pore sized nylon filter. Retained material
was resuspended in HAM’s F12 and prepared for scanning electron
microscopy.

Scanning Electron Microscopy—For scanning electron microscopy
(SEM), 50 μl of the final suspension was gently drawn onto a wet
Millipore filter, pore size 0.22 μm. The whole filter was fixed in 2.5%
glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4, for two
hours at 4 °C. Alternatively, filters were fixed in 2% paraformalde-
hyde, 0.2% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4, for 1 h
at 4 °C, pre-incubated with 0.5% BSA in the same buffer for 1 h at
room temperature and subsequently incubated with an antiserum raised
against bovine collagen VI for 1 h at room temperature, washed with 0.1 M
sodium cacodylate, pH 7.4, and incubated with protein A-gold as
described (43). Fixed filter samples were dehydrated for 10 min at each step of an
ascending ethanol series and critical point dried in a Balzers critical
type dryer. They were mounted on aluminum stubs, gold/palladium
coated and examined in a Jeol J-3300 scanning electron microscope.

Alternatively, immunolabeled filters were coated with a 20 nm carbon
layer under rotation prior to SEM. Gold label was detected by recording
back scattered electron images at an acceleration voltage of 30 kV and
a working distance of 10 mm.

Double Immunolabeling of Chondrosarcoma Cells—The Swarm rat
chondrosarcoma cell line (44) was a kind gift from Dr. J. Kimura (Henry
Ford Hospital, Detroit). The immortalized cells were cultured in HAM’s
F12 supplemented with 10% fetal calf serum, 50 units/ml penicillin, 50
units/ml streptomycin, and 50 μg/ml l-ascorbic acid.

For immunolabeling the cells were fixed for 20 min with 3.6% for-
aldehyde in phosphate-buffered saline. Nonspecific antibody binding
was blocked by incubation for 30 min with 1% (w/v) BSA in PBS. Immunolabelling
was done by consecutive treatment of the slides for 1 h with a mixture of the two appropriate primary antibodies and a mixture
of the two secondary antibodies. As secondary antibodies a Cy3-
conjugated donkey anti-chicken IgG (Jackson Immuno) or a Cy3-
conjugated affinity-purified anti-rabbit IgG (Jackson Immuno) and a Cy3-
conjugated affinity-purified anti-mouse IgG (Jackson Immuno) were used in combination with either a Cy3/Cy5-conjugated affinity-puri-
fied goat anti-rabbit IgG (Jackson Immuno) or a Cy3/Cy2-conjugated
affinity-purified goat anti-mouse IgG (Jackson Immuno). All antibodies
were diluted in 1% (w/v) BSA in PBS.

Purification of Protein Complexes from the Swarm Rat Chondrosar-
coma—Tissue was washed with five volumes of ice cold 0.05 M Tris-HCl,
0.15 M sodium chloride pH 7.4 (TBS) containing, 5 mM calcium chloride
and extracted twice for 1 h and once overnight at +4 °C in TBS con-
taining 10 mM EDTA (45). All buffers contained protease inhibitors (46).

The tissue residues were removed by centrifugation at 10000 × g for 20
min and the supernatant was centrifuged at 100 000 × g for 8 h at
+4 °C (Beckman 70.2 Ti). The bottom fraction, enriched in high mol-
ecular weight components, was dissolved in TBS at +4 °C for 48 h.
Insoluble material was pelleted by centrifugation at 10 000 × g for 30
min. Protein complexes in the supernatant were purified further by rate
zonal centrifugation in a 12 ml glycerol gradient (10–50%) for 3 h at 200
000 × g in a Beckman SW41 Ti rotor. The tube content was divided into
equal fractions and their molecular constituents were analyzed by
electron microscopy after negative staining.

Preparation of Gold-labeled Proteins and Antibodies—Colloidal gold
particles of 4 nm ± 15% were prepared by reduction of HauCl4 by
thiocyanate (47) and conjugated after titration to polyclonal antibodies
(against human proN(II)-peptide and murine matrilin-2). Polyconal,
affinity-purified antibodies (against bovine biglycan, decorin, chon-
droadherin, and matrilin-1, and against murine matrilin-3 and matrilin-4)
and monoclonal antibodies (against human aggrecan). In some experi-
ments, colloidal gold particles with a larger (12 ± 20%) diameter were
prepared by reduction of HauCl4 using sodium citrate (48) and conju-
gated after titration to the above-described antibodies against matri-
lin-1. The middle fraction from the rate zonal centrifugation was diluted
into TBS and incubated with different dilutions of gold-labeled antibod-
ies. As a control, samples were incubated with pre-immune serum
under the same conditions. Samples were adsorbed to a 400-mesh
carbon coated copper grid, which was rendered hydrophilic by glow-
 discharge at low pressure in air. The grid was immediately blotted,
was washed with two drops of water, and stained with 0.75% uranyl
formate for 15 s. Samples were observed in a Jeol 1200 EX transmission electron
microscope operated at 60 kV accelerating voltage and ×75,000 magnifi-
cation. Evaluation of the data from electron micrographs was done
as described previously (49).

Antibody specificity was determined by SDS-polyacrylamide gel

electrophoresis and immunoblotting. Samples of TBS/EDTA extracts from
chondrosarcoma tissue were digested with chondroitinase ABC prior to
electrophoresis by incubation with 50 microunits/ml chondroitinase
ABC (Seikagaku). Samples were allowed to react at 37 °C for 1–2 h,
subjected to ethidium precipitation and resolved in sample buffer with
5% β-mercaptoethanol. SDS-polyacrylamide gel electrophoresis was
performed as described by Laemmli (50). For immunoblots were
electroblotted to nitrocellulose membranes and incubated with a
dilution of the appropriate antibody. Bound antibodies were detected by
using peroxidase-conjugated swine anti-mouse IgG, 3-aminophthalhy-
drazide (1.25 mm), p-coumaric acid (225 μM), and 0.1% H2O2.

Interactions Studied by Surface Plasmon Resonance in the BIA-
core™2000 System—Matrilin-1, purified from native extracts of bovine
fetal cartilage (51) (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-dimethylaminopro-
pyl)carbodiimide and N-hydroxysuccinimide (Sigma-Aldrich) according
to the description of the manufacturer. Remaining active groups on the
surface were blocked with 1 M ethanolamine.HCl 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).

Studies of interactions were performed in TBS, pH 7.4, containing
0.05% surfactant P20. Native recombinant biglycan, decorin, and
chondroadherin, produced in the human 293 cell line (20–200 μM) were
injected over immobilized matrilin-1 at flow rates of 40–50 μl/min.

Collagen VI Microfibrils—Collagen VI microfibrils without bound
protein complexes were prepared from bovine cornea (52). In short,
bovine corneas were cut into pieces and homogenized in Tris/saline
buffer, pH 7.4, containing 5 mM calcium chloride and protease inhibi-
tors. The homogenate was digested with collagenase type 1 (Worthing-
ton) for 1 h at 37 °C and dissolved material was washed by centrifugation at 48 000 ×
g for 20 min. The supernatant was applied onto a 25 ml Superose 6 column (Amersham Biosciences) equilibrated and eluted with
homogenization buffer at 0.2 ml/min. Fractions of 0.5 ml from the void
volume containing collagen VI microfibrils were collected and pooled.

Recombinant LRR-Protoglycan/Proteins—Preparation of native recombi-
nant LRR-protoglycans (LRR-PrG) has been described by several groups in
to human kidney cells, human HeLa cells, and Chinese hamster ovary cells has been previously
described (41).

Reconstitution of Complexes in Vitro—Colloidal gold particles were
prepared through reduction with thiocyanate (smaller size) or citrate
(larger size) as described above. Colloidal gold particles of smaller and
larger size were titrated and conjugated to biglycan, decorin, chondo-
droadherin, and matrilin-1, respectively. Gold-labeled proteins
were incubated with purified collagen VI microfibrils from cornea, incubated
for 15 min in +4 °C and then subjected to negative staining and elec-
tron microscopy as described above.
RESULTS

Structural Analysis of the Pericellular Matrix of Chondrons and Cultured Chondrocytes—Chondrons, representing single or clustered chondrocytes surrounded by territorial matrix, were purified in an intact form from Swarm rat chondrosarcoma tissue using a modification of a method previously reported for mature cartilage (42). The high water content and the softness of the tissue allowed isolation of the chondrons by passing tissue samples through nylon filters. Chondrons isolated by this method exhibited the same overall appearance as those isolated from cartilage by Poole et al. (42) (Fig. 1a). SEM analysis showed that the chondron capsule contains different fibrillar networks where thin microfibrils contrast against thicker fibers (Fig. 1b). The thinner fibrils where identified as collagen VI by immunolabeling and SEM (Fig. 1c). Cultured Swarm rat chondrosarcoma cells deposit a pericellular matrix containing collagen VI microfibrils similar to those found in vivo (Fig. 1, d–f). Immunofluorescence microscopy of the chondrosarcoma cells showed co-localization of collagen VI microfibrils with both matrilin-1 and matrilin-3 (Fig. 2). Since the two matrilins showed extensive co-localization with each other and may well occur in hybrid molecules and hybrid filaments (21, 26), only matrilin-3 was studied in further double immunofluorescence experiments (Fig. 2). Biglycan and decorin showed localizations partially overlapping with matrilin-3 in pericellular filaments. In addition, matrilin-3 was partially co-distributed with both aggrecan and collagen II.

To assure that all antibodies used were truly specific, these were tested in immunoblots against extracts of chondrosarcoma proteins and proteoglycans that had been resolved by SDS-PAGE performed under reducing conditions. All antibodies reacted specifically with bands with a mobility characteristic for the corresponding antigen (not shown).

Analysis of Isolated Collagen VI Microfibrils by Electron Microscopy—Collagen VI microfibrils were obtained in an intact form by extraction of rat chondrosarcoma tissue with EDTA-containing physiological saline, followed by a differential centrifugation procedure. The fibrils were examined by electron microscopy after negative staining. Collagen VI was visualized as beaded filaments where adjacent tetramers were linked together in the characteristic end-to-end manner (Fig.
3, a and i). These filaments formed complexes with a number of molecules giving their N termini a bulky appearance (Fig. 3a, arrow) compared with the collagen VI microfibrils without bound ligands (Fig. 3h, arrow). The latter filaments were prepared by collagenase digestion of bovine cornea (52). Two different kinds of particles were frequently observed at the N termini of collagen VI filaments derived from the chondrosarcoma (Fig. 3a, insets). The smaller of these consisted of two globular domains connected by a smaller, central domain. The larger was in most cases composed of three globular domains of equal size (Fig. 3a, insets), but also structures made up of four subunits were frequently seen. The molecular identity of the particles was determined using gold-labeled, affinity-purified antibodies. Using this approach, biglycan (Fig. 3b), and decorin (Fig. 3c) were found located to the N-terminal globular domains of collagen VI and represented the smaller kind of particle. The larger molecules, typically bound to the small LRR-proteoglycans, and more distant from the collagen VI filaments, were identified as matrilin-1 (Fig. 3d), matrilin-3 (Fig. 3e), and matrilin-4 (Fig. 3f). Matrilin-2 was never detected as a component of the collagen VI microfibril complexes (not shown). We also identified chondroadherin bound both to the N- and C-terminal globular domains of collagen VI, but we did not observe other proteins interacting with chondroadherin in these samples (Fig. 3i).

Reconstitution of Collagen VI Complexes in Vitro—The observations made on native, chondrosarcoma-derived collagen VI complexes were verified in recombination experiments employing highly purified proteins and recombinant native proteoglycans (Fig. 4). Corneal collagen VI microfibrils, free of associated proteins, were mixed with LRR-proteoglycans/proteins in vitro. Recombinant native biglycan, decorin, and chondroadherin were labeled with colloidal gold prior to incubation. Biglycan and decorin bind exclusively to the N-terminal parts of collagen VI, while chondroadherin was not detected as a component of the collagen VI microfibril complexes (not shown). We also identified chondroadherin bound both to the N-and C-terminal globular domains of collagen VI, but we did not observe other proteins interacting with chondroadherin in these samples (Fig. 4).
to the collagen VI via biglycan (Fig. 4d) or decorin (Fig. 4e), labeled with smaller colloidal gold, when these molecules were mixed prior to incubation with the corneal collagen VI microfibrils.

Analysis of the Interactions between Matrilin-1 and LRR-proteoglycans—The results obtained by electron microscopy indicated that matrilin-1 is able to bind the LRR-proteoglycans biglycan and decorin. This was further supported in binding experiments using purified proteins and proteoglycans and detecting bound proteins by surface plasmon resonance in the BIAcore™2000 system. Matrilin-1 was immobilized to the CM-5 chip and biglycan, decorin or chondroadherin added in the fluid phase (Fig. 5). For biglycan and decorin a concentration-dependent binding to matrilin-1 was observed, while chondroadherin did not interact. The lack of binding of chondroadherin to matrilin-1 is in agreement with the results from electron microscopy, where this LRR-protein was never seen associated with matrilsins (Fig. 3g). The binding of native biglycan and decorin to matrilin-1, but not of the structurally related chondroadherin, demonstrates the specificity of the assay.

Identification of Extracellular Matrix Components Interacting with Collagen VI Microfibrils via the LRR-proteoglycan/matrilin Complex—In electron microscopy, the LRR-proteoglycan/matrilin complexes were frequently seen to mediate contacts between the collagen VI filaments and other matrix macromolecules. These peripherally attached molecules were identified as procollagen II (a–c) and aggrecan (d and e) networks were found connected to collagen VI microfibrils via the matrilin-1/LRR-proteoglycan complex. Gold-labeled antibodies (white arrows) were used to directly identify procollagen (a), matrilin-1 (b and e) and the aggrecan core protein (d) in purified high molecular complexes. Arrowheads in (b and e) point to matrilin-1 consisting of three globular domains. In (a and d) arrowheads point to similar particles consisting of three globular domains, resembling matrilsins. c, double staining with gold of different sizes occasionally located complexes of biglycan (white arrow, small gold) and matrilin-1 (black arrow, large gold) between collagen VI microfibrils and striated collagen II fibrils. Note that the enlargement in c is different from (a, b, d, e). The bars represent 100 nm.
Supramolecular assembly of collagen VI molecules into fibrillar networks has been evaluated in detail previously (3, 4, 7), but how these networks are associated with other interstitial network components at the molecular level is still elusive. Although collagen VI has been shown to interact with fibrillar collagens in vitro (6, 34) it has been suggested that other molecules may be involved in the in vitro interaction (53).

In the present study we provide evidence for molecular mechanisms by which collagen VI microfibrils can interact with other interstitial extracellular matrix networks. We took advantage of the Swarm rat chondrosarcoma, a comparatively soft cartilaginous tissue, which allows purification of cartilage extracellular matrix components under native conditions. Electron microscopy in combination with negative staining and colloidal gold technology was used to visualize the extracellular matrix structures at high resolution and to identify individual components both in native extracts and reconstituted multimolecular assemblies. For the recombination experiments bovine proteins were used. Identical results were obtained when bovine matrilin-1, CHAD, biglycan, and decorin were used in combination with rat collagen VI (not shown). Despite the species difference the results did in all aspects agree with and confirm the observations made in the Swarm rat chondrosarcoma system.

Compared with “naked” beaded microfibrils extracted by limited collagenase digestion (52) the collagen VI microfibrils, extracted under conditions that preserve native structures, exhibited a variety of globular multidomain proteins surrounding the globular beads of the tetramer junctional complex. Gold-labeled antibodies confirmed that the smaller particles, binding close to the microfibril, were different LRR proteins or proteoglycans, whereas the larger particles found more distant from the microfibril were identified as different matrilins. Biglycan and decorin were found in the vicinity of the N terminus, whereas chondroadherin bound close to both the N and C termini. In vitro reconstituted complexes consisting of collagenase-digested microfibrils with added gold-labeled biglycan, decorin, or chondroadherin, looked similar. This is in accordance with previous results showing in vitro binding of biglycan and decorin close to the N-terminal region of collagen VI tetramers (35), and chondroadherin to both N- and C-terminal globular moieties (41).

Native glycosylated biglycan and decorin, but not chondroadherin, interacted with matrilins in both native and reconstituted microfilar assemblies. The interactions were not dependent on glycosaminoglycan chains as neither pretreatment of native biglycan and decorin with chondroitinase ABC, nor the presence of purified chondroitin or dermatan sulfate chains inhibited the assembly (results not shown). Complexes between matrilin-1 and biglycan/decorin were examined in further detail as models for this interaction, where different complexes between LRR-proteoglycans and matrilins were found bound to native collagen VI microfibrils. The finding, that collagen VI is connected to the major constituents of extracellular matrix through the LRR-proteoglycans in complex with the matrilins, is in accordance with the fact that matrilin-1 interacts with both aggrecan and collagen II (27–29). By use of electron microscopy we visualized the individual molecules and their interrelations in native complexes extracted from tissue and demonstrate that the major structural entities, collagen II and aggrecan are joined to collagen VI via a complex of matrilins and LRR-proteoglycans. This observation offers new insights into the complex organization of the cartilage matrix and allows us to propose a model of the in vivo organization of the studied molecules (Fig. 7). We conclude that an important role of matrilin-1, biglycan and decorin is to serve as adapter proteins connecting macromolecular networks in the cartilage extracellular matrix. The members of the matrilin family are differentially expressed among and within cartilages. For example, the superficial zone of articular cartilage lacks matrilin-1 and -3 while matrilin-4 is present (54). It is likely that matrilin-4 takes over the adapter function in this tissue, but it may be that the levels of matrilin expression influence the kind of macromolecular assemblies formed in a particular tissue compartment. Other members of the matrilin and LRR-proteoglycan families may play similar roles in the tissues where they are expressed.

Our study visualizes collagen VI microfibrillar networks, which are connected to collagen II fibrils by biglycan/matrilin-1 complexes as linkers. These linkers also connect a number of procollagen molecules to the collagen VI scaffold. These may represent immobilized nucleation centers for collagen II fibril assembly. It is thus possible that a functional role of such collagen VI microfibril supramolecular assemblies is to act as scaffolds for the formation of the structurally critical fibrillar collagen networks. A further role of the collagen VI network may be to present fibrillogenesis modulators such as LRR proteins in proximity to the growing fibrils. In this way the formation of the collagen VI microfibrillar network in the early stages of tissue formation, or in repair processes such as wound and fracture healing, may play an important instructional role in tissue development, architecture, and homeostasis.

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