Association of the Aggrecan Keratan Sulfate-rich Region with Collagen in Bovine Articular Cartilage*

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Aggrecan, the predominant large proteoglycan of cartilage, is a multidomain macromolecule with each domain contributing specific functional properties. One of the domains contains the majority of the keratan sulfate (KS) chain substituents and a protein segment with a proline-rich hexapeptide repeat sequence. The function of this domain is unknown but the primary structure suggests a potential for binding to collagen fibrils. We have examined binding of aggrecan fragments encompassing the KS-rich region in a solid-phase assay. A moderate affinity (apparent $K_d = 1.1 \mu M$) for isolated collagen II, as well as collagen I, was demonstrated. Enzymatic digestion of the KS chains did not alter the capacity of the peptide to bind to collagen, whereas cleavage of the protein core abolished the interaction. The distribution of the aggrecan KS-rich region in bovine tarsometatarsal joint cartilage was investigated using immunoelectron microscopy. Immunoreactivity was relatively low in the superficial zone and higher in the intermediate and deep zones of the uncalcified cartilage. Within the pericellular and territorial matrix compartments the epitopes representing the aggrecan KS-rich region were detected preferentially near or at collagen fibrils. Along the fibrils, epitope reactivity was non-randomly distributed, showing preference for the gap region within the $d$-period. Our data suggest that collagen fibrils interact with the KS-rich regions of several aggrecan monomers aligned within a proteoglycan aggregate. The fibril could therefore serve as a back-bone in at least some of the aggrecan complexes.

Articular cartilage matrix can be regarded as a fiber-reinforced composite material (1), where aggrecan complexes are entangled within a network of collagen fibrils. The aggrecan complexes, constituting about 90% of the proteoglycan content (2), endow the matrix with high osmotic pressure, compressive stiffness, and resilience, whereas collagen is essential for the tensile strength of the tissue (3). Different mechanical properties of the composite depend on these major constituents and how they are assembled and stabilized by intermolecular interactions. The capacity of cartilage to withstand mechanical stress depends upon its structural integrity and, hence, numerous interactions between the matrix components. Indeed, the molecules are so tightly associated that most of the tissue constituents require denaturing solvents or proteases for extraction. This has hampered studies of molecular function. To gain insight into the physiology of articular cartilage, it is necessary to identify and characterize interactions between the matrix constituents, particularly those involving the collagen.

In the present study we focused on a domain of the aggrecan molecule containing the majority of the keratan sulfate (KS) chain substituents and a protein segment with a hexapeptide repeat sequence (4). The second globular domain (G2) is localized adjacent to this KS-rich region, on the N-terminal side. The first globular domain (G1), which represents the hyaluronan-binding region (HABr), is localized next to G2 as the very N-terminal portion of aggrecan. On the C-terminal side of the KS-rich region, there is a large chondroitin sulfate (CS)-rich region that accounts for more than 80% of the molecular mass. The very C-terminal end contains a number of distinct domains including one with homology to C-type lectins.

Proteoglycan aggregates are made up of several aggrecan molecules that are bound via their G1 domains to hyaluronan. The binding is stabilized by a third component, the link protein, that binds simultaneously to aggrecan and hyaluronan (see Heinegård and Sommarin, Ref. 5). In the aggregate, the G2 and KS-rich portions are interspaced between the central hyaluronan strand and the region with the densely packed CS chains. The structure of the KS-rich region suggests a relatively extended and rigid conformation. This is supported by electron microscopy of spread proteoglycan aggregates after rotary shadowing, showing that the CS-rich region is clearly separated from the central filament of hyaluronan (6). It appears that the KS-rich region acts as a spacer within the proteoglycan aggregate. Interestingly, the dimensions of this region are similar to the diameter of the collagen fibril.

The primary structure of the KS-rich region polypeptide encompasses a number of consecutive proline-rich hexapeptide...
Affinity purified antibodies were incubated enzymatically generated cleavage products of aggrecan, in microplate wells coated with aggrecan core protein. Inhibition of followed by dialysis against 0.15 M NaCl, 5 mM NaPi, pH 7.4. a substrate for bound enzyme.  

Conjugate (Orion Chemicals), followed by paranitrophenyl phosphate as by incubation with a swine anti-rabbit (IgG) alkaline phosphatase conjugate and paranitrophenyl phosphate as a substrate for bound enzyme.

The proline-rich repeat may form a polyproline coil. Such a structure has the potential for interacting with the collagen fibril, which in this case could find sufficient space to run through the center of the proteoglycan aggregate and by interactions reinforce the network of assembled aggrecan molecules. This hypothesis was put to test in the present work.

**EXPERIMENTAL PROCEDURES**

**Proteoglycan Fragments**—Fragments of bovine nasal cartilage aggrecan were prepared according to previously described procedures (7). The tryptic-resistant fragment KS.t represents the KS-rich region extended to and including parts of the G2 domain (8). A more extensively trimmed fragment, KS.t.c, which was obtained by additional digestion with chymotrypsin, represents exclusively the KS-rich region (7). The tryptic fragment HABr represents the hyaluronan-binding region, whereas the preparation CS pep contains several peptides derived from the CS-rich region. Intact aggrecan core protein devoid of CS was prepared by digestion with chondroitinase ABC (9).

**Purification of Antibodies**—The antibodies against the KS-rich portion of aggrecan were prepared from a polyclonal antiserum raised against the entire bovine aggrecan molecule (10). Two affinity columns containing CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech), to which isolated aggrecan fragments had been coupled, were used for the purification. The antiserum was first passed through a column containing the fragment HABr coupled at 2 mg/ml of gel. The flow-through fraction of the antiserum, thus devoid of unspecifically binding antibodies or antibodies recognizing epitopes within the HABr, was subsequently passed through a column containing gel with coupled KS.t. Bound antibodies were eluted with 3M potassium isocyanate (Seikagaku Corp.). Samples of KS.t and KS.t.c were digested with pepsin-extracted collagen I at 10 \( \mu \text{g/mL} \) or collagen II at 50 \( \mu \text{g/mL} \) in 0.14 \( \text{m NaCl} \), 30 \( \text{mM NaPi} \), pH 7.3, and after that coated for 1 h with \( \alpha \)-casein at 100 \( \mu \text{g/mL} \) to block unspecific interactions. The wells were washed with buffer containing 0.05% Tween 20 and then incubated with ligand solution. Antibodies used for detection of bound ligand were either the affinity purified anti-KS.t antibodies or the monoclonal 5D4 that specifically recognizes KS (12). These primary antibodies were detected with a secondary antibody, the former with a swine anti-rabbit (IgG) alkaline phosphatase conjugate and the latter with a goat anti-mouse (IgG) (Orion Chemicals), and the substrate paranitrophenyl phosphate. Absorbance was measured at 405 nm, and the data were processed as described (11).

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Before some of the binding experiments, KS was digested using either endo-\(\beta\)-galactosidase (Boehringer Mannheim) or keratanase II (Seikagaku Corp.). Samples of KS.t and KS.t.c were digested with approximately 5 million units of enzyme/mg of substrate in 0.14 \( \text{m NaCl} \), 30 \( \text{mM NaPi} \), pH 7.3, for 2 h at 37 °C. Efficiency of digestion was confirmed by SDS-polyacrylamide gel electrophoresis, showing higher migration rates of fragments after enzyme treatment.

In one set of experiments, the binding of KS.t after papain cleavage...
inactivated when added to the proteoglycan fragments (●). Alternatively, the KS.t samples contained papain that already was digested at 40 °C for 24 h. The enzyme was then inactivated by addition of iodoacetamide to a concentration of 20 mM and heating in a boiling water bath for 5 min. A control sample was prepared from another area of the pericellular and territorial compartments and a ratio between fibrillar and interfibrillar labeling was calculated for each compartment. Fibrillar labeling was defined as gold particles projected over collagen fibrils. Interfibrillar labeling was defined as all that outside the border of the fibril. The number of gold probes was counted, and the collagen volume density (v/v) in the pericellular and territorial compartments of zone II was measured by point counting (16). Immunoreactivity was then correlated to collagen v/v. Immunoreactivity was visualized with protein-A conjugated to 10-nm gold probes (Amersham Pharmacia Biotech, UK) in sections contrasted with uranyl acetate and lead citrate.

For assessing the association between collagen fibrils and labeling (n = 3), matrix was divided into two compartments. Fibrillar labeling was defined as gold particles projected over collagen fibrils. Interfibrillar labeling was defined as all that outside the border of the fibril. The number of gold probes was counted, and the collagen volume density (v/v) in the pericellular and territorial compartments and a ratio between fibrillar and interfibrillar labeling was calculated for each compartment.

To study the distribution of the aggrecan KS-rich region along the collagen fibrils, bovine tarsometatarsal joint cartilage (n = 3) was prepared for cryosectioning as described elsewhere (15). Ultrathin cryosections (100 nm) were incubated with primary antibodies and immunoreactivity was detected with protein-A-coated 10-nm gold probes. From random high power (×250,000) electron micrographs, the distribution of gold probes along the fibrils was measured (15).

RESULTS

Specificity of Antibodies—Binding of affinity purified antibodies to the aggrecan core protein was measured by enzyme-linked immunosorbent assay. To determine the localization of epitopes within the aggrecan structure, inhibition of binding by different aggrecan fragments was examined (Fig. 1). Neither isolated HABr nor peptides representing the CS-rich region inhibited the binding of antibodies. Two different preparations of fragments containing the KS-rich region strongly inhibited binding. The fragments prepared using trypsin in combination with chymotrypsin inhibited the antibody binding to the same extent as the fragments prepared using trypsin only. Because


**Table I**

| Zone | Pericellular | Compartment | Interterritorial |
|-----|--------------|--------------|-----------------|
| I   | 1.19 (0.50)  | 1.14 (0.43)  | 1.07 (0.52)     |
| II  | 2.54 (0.65)* | 2.37 (0.61)* | 2.05 (0.73)*    |
| III | 2.59 (1.06)* | 2.51 (0.98)* | 1.77 (0.71)     |

* indicates significant difference between zone I and II; ** indicates significant difference between zone I and III, p < 0.05.

**Table II**

**Correlation of aggrecan keratan sulfate-rich region to collagen fibrils in the pericellular and territorial compartments of zone II in bovine articular cartilage.**

| Compartment | Pericellular | Territorial |
|-------------|--------------|-------------|
| Collagen volume density | 0.32 (0.05) | 0.48 (0.06) |
| Fibrillar labeling | 64.0 (12.8) | 47.4 (13.1) |
| Interfibrillar labeling | 16.0 (2.1) | 18.4 (2.8) |
| Ratio fibrillar/interfibrillar labeling | 4.0 (0.4) | 2.5 (0.5) |



The fragments remaining after chymotrypsin digestion essentially represent the KS-rich region proper, i.e., the hexapeptide repeat structure with the KS chains, the antibodies used here mainly recognize epitopes within this region.

**Binding of Aggrecan KS-rich Fragments to Collagens in Vitro**—As discussed above, the KS-rich region has a structure indicative of a potential for binding to collagen. To examine this possibility, we studied binding of the isolated domain to collagen in vitro. A microplate solid-phase binding assay was used. The fragment KS.t, which contains the KS-rich region and the polypeptide up to and in some fragments actually including parts of the second globular domain, showed saturable binding to collagen I as well as collagen II (Fig. 2, top). The amounts of KS.t bound at saturation were larger for wells with collagen I than for wells with collagen II, probably reflecting differences in the amounts of immobilized collagen. In a Scatchard-type plot, the data displayed linear correlations, indicating that the binding sites were homogeneous (Fig. 2, bottom). The affinity of the KS.t fragment was the same for collagen I and collagen II, with an apparent dissociation constant of 1.1 μM. This represents a fairly weak interaction. For comparison, the affinities shown by the small proteoglycans decorin and fibromodulin in the same type of assay were about 100 times higher (11).

Binding of the fragment KS.t.c, apparently representing the repeat polypeptide with the KS-chains only (7), to collagen I was not different from that of KS.t (Fig. 3). Hence, the peptide portions adjacent to the repeat defining the true KS-rich region did not contribute significantly to the collagen interactions. Moreover, the fragments still had capacity to bind to collagen after enzymatic hydrolysis of the KS (Fig. 3) using either keratanase II or endo-β-galactosidase. However, the binding capacity was dramatically reduced if the KS.t core protein was extensively cleaved by digestion with papain (Fig. 4). These observations support the view that the interaction depends on the core protein.

To further characterize the collagen interaction of the KS.t fragment, the sensitivity to increased ionic strength was examined. Binding was not affected by small differences in salt concentration within the near-physiological range, but it was gradually decreased at NaCl concentrations higher than 0.15 M (Fig. 5). This suggests that charged groups are important for the interaction. The primary structure of the KS.t core protein consists of repeated hexapeptide units, typically with the sequence Glu-Xaa-Pro-Phe-Pro-Ser (4). Glutamic acid residues occupy the first position in 22 of the 23 units and, in addition, the second position in 11 of the units. Therefore, it is likely that the negative charges of the glutamates have a role in the interaction. Still, the observation that significant binding occurred at the rel-
the fibril axis. Labeling showed a marked preference to the gap the KS-rich region displayed a non-random distribution along labeling than that obtained with sections of resin-embedded assessed with cryosections at high power magnification. Cryo-

territorial matrix. This was because of the high volume density of consequently a major proportion of the probes within this dis-

targets on the collagen fibril to be projected outside the fibril. gold complex allow a proportion of the gold particles attached to true fibrillar labeling because the dimensions of the immuno-
tangent to the border of the fibril were counted as fibril-asso-

ments (Table II). Only probes projected on a collagen fibril or fibrillar labeling in the pericellular and territorial compart-

ments closer to the cells. Because it is likely that all aggrecan proteoglycans are bound to the fibrils by strong interactions, in

immunogold-labeling with the antibodies, showed that reactiv-

ity was lowest in the superficial part and about two times higher in the middle and deep parts (Table I). Labeling of the interterritorial matrix was slightly lower than that for the matrix closer to the cells. Because it is likely that all aggrecan molecules retained in the matrix will contain the KS-rich region, regardless of partial fragmentation, the distribution of the KS.t immunoreactivity can be taken as a measure of aggrecan distribution. The results corroborate and extend those obtained by other techniques (17–19).

Immunoreactivity for the aggrecan KS-rich region was prefer-

entially localized within the proximity of collagen fibrils. Thus, fibrillar labeling was about 2–4 times higher than inter-

fibrillar labeling in the pericellular and territorial compart-

ments (Table II). Only probes projected on a collagen fibril or
tangent to the border of the fibril were counted as fibril-asso-

iated (Fig. 6). This should represent an underestimation of the true fibrillar labeling because the dimensions of the immuno-

gold complex allow a proportion of the gold particles attached to targets on the collagen fibril to be projected outside the fibril. The expected diameter of the complex is about 23 nm (20), and consequently a major proportion of the probes within this dis-
tance from the fibril surface may reflect the fibril-associated KS-rich region. However, we refrained from regarding any probes not touching the fibrils as associated because this would have imposed difficulties in defining the interfibrillar matrix. Unfortunately, it was still not possible to distinguish between fibrillar and interfibrillar labeling on micrographs showing interfibrillar matrix. This was because of the high volume density of fibrils and concomitant overprojection of gold particles.

The distribution of gold probes along the individual fibril was assessed with cryosections at high power magnification. Cryo-

sections were preferred because they give a higher intensity of labeling than that obtained with sections of resin-embedded tissue. In this case, immunolabeling with antibodies against the KS-rich region displayed a non-random distribution along the fibril axis. Labeling showed a marked preference to the gap region within the D-period (Fig. 7). A more precise identifica-
tion of the localization within the gap region is not feasible with the present technique because of limitations imposed by the size of the immunogold complex, which represents about one-

third of the length of the D-period (20).

DISCUSSION

Previous histochemical studies have indicated a close spatial relationship between proteoglycans and collagen in soft tissues, e.g. in tendon and annulus fibrosus (21) and in articular carti-
lage (22). Immunohistochemical examination has shown that the small dermatan sulfate proteoglycan decorin is associated with fibrils in tendon (23) and dermis (24), apparently because of interactions between the core protein and collagen. In carti-
lage, both decorin and collagen IX occur as fibril-associated proteoglycans (25, 26), albeit that most of the collagen IX molecules in mammalian cartilage do not appear to have an attached glycosaminoglycan chain (27). Furthermore, we have observed that the keratan sulfate proteoglycan fibromucin is also distributed along collagen fibrils in articular cartilage (15), also apparently bound via its core protein (11, 28). These small proteoglycans are bound to the fibrils by strong interactions, in the case of collagen IX stabilized by covalent bonds. Therefore, these molecules can be considered as fibril constituents. In contrast, the large cartilage proteoglycan aggrecan occurs in

the tissue in a well characterized supramolecular form that is distinct from the fibrils. This aggregate consists of up to 100 aggrecan monomers and an equal number of link proteins, associated with a single strand of hyaluronan. It is likely that there are interactions between collagen fibrils and the proteo-
glycan aggregates, important for the mechanical properties of cartilage, but the mechanisms are not known. Based on work with in vitro systems, it has been proposed that the CS-rich region of aggrecan is essential for interactions (29, 30). The existence of an interaction of some kind within the tissue, is supported by observations made by immunoperoxidase elec-
tron microscopy indicating a periodic arrangement of the proteo-
glycan along cartilage fibrils (31).

The results presented here provide strong evidence that a proteoglycan aggregate interacts with a collagen fibril located within the aggregate, near the central hyaluronan strand. It is reasonable to assume that the KS-rich region represents an extended structure surrounded by free space. This molecular architecture may allow diffusion of some matrix constituents, e.g. procollagen, and may participate in specific interactions. Hypothetically, a collagen fibril may be positioned as a back-

bone within the aggrecan complex and connect several aggrecan KS-rich regions (Fig. 8). Even though each KS-rich region forms a fairly weak interaction, the multiplicity existing in the aggregate would result in a very tight complex with the colla-

gen fibril. Such an arrangement would considerably influence the structural integrity of the proteoglycan aggregate. It could also have an important role in providing the microenvironment for the formation of fibrils in cartilage.

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