Interaction of Cartilage Matrix Protein with Aggrecan

INCREASED COVALENT CROSS-LINKING WITH TISSUE MATURATION*

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Cartilage matrix protein (CMP) is a trimeric protein present in many types of cartilage extracellular matrix. It has recently been purified under native conditions by Hauser, N., and Paulsson, M. (1994) J. Biol. Chem. 269, 25747–25753. To examine the functional properties of CMP we studied its interaction with aggrecan within cartilage extracellular matrix. Aggrecan-enriched fractions were purified from bovine tracheal cartilage of different ages under non-denaturing and denaturing conditions, respectively, and characterized by a combination of biochemical methods and electron microscopy. The fractions contained a pool of CMP non-covalently associated with aggrecan as well as a pool of CMP that appears covalently cross-linked to the aggrecan core protein. Only about two thirds of the CMP subunits could be released even upon reduction under denaturing conditions. It appears that CMP is attached by a non-reducible covalent interaction of one of its subunits with the protein core. The amount of CMP strongly bound to aggrecan increases with age. Electron microscopy revealed interaction sites for CMP in the extended chondroitin-sulfate attachment domain E2. In old tissue five distinct binding sites for CMP were found while in young cartilage only three of these were occupied. The extent of decoration of E2 with CMP increases with age.

The molecular composition and supramolecular structure of cartilage determines the physical properties essential for its function. The cartilage tissue consists of chondrocytes surrounded by an abundant extracellular matrix. This matrix is composed of collagens (1), proteoglycans (2, 3), and non-collagenous matrix proteins (4). These have been partially characterized but the knowledge of their interactions and physiological functions is still fragmentary.

Under associative conditions, the major large proteoglycan in cartilage, aggrecan, can be isolated as aggregates formed by the noncovalent interaction of aggrecan monomers with hyaluronate and link protein. A combination of biochemical and biophysical characterization with interpretation of sequence information and electron microscopy allowed the proposal of a detailed model for aggrecan (see Ref. 5 and references therein). The core protein consists of two N-terminal globular domains G1 and G2, interspaced by an extended domain E1, followed by a long extended domain E2, which is terminated by the C-terminal globular domain G3. G1 corresponds to the hyaluronate-binding region (6, 7). G2 shows a high degree of sequence homology to G1, but lacks affinity for hyaluronate (7, 8). E2 carries the bulk of glycosaminoglycan side chains. In its N-terminal part it represents the keratan-sulfate-rich region substituted with some two thirds of the total keratan sulfate in a very dense arrangement (9). This polypeptide, being extremely rich in glutamate and proline (10), consists of 23 hexapeptide repeats in bovine cartilage aggrecan but only 4 in rat chondrosarcoma aggrecan (11, 12). The C-terminal part of E2 constitutes the chondroitin-sulfate-rich region accounting for the major part of the core protein. It contains most of the chondroitin sulfate chains and some of the keratan sulfate (9). The chondroitin sulfate is arranged in clusters (13) and sequence analysis shows that this segment of E2 can be divided into several subdomains all containing the putative Ser-Gly glycosylation sites, but differing with respect to the length of internal repeats (11). It appears that all of the Ser-Gly sites are substituted with chondroitin sulfate chains as their number closely equals the number of side chains determined by biochemical analysis. E2 is of variable length (6, 14), apparently due to proteolytic degradation within the matrix. Its average length in the intact monomer is 405 nm, but only 263 nm in the chondroitinase digested core protein fragment, implying a stretching effect by glycosaminoglycan repulsion in intact monomers (15). The C-terminal globular domain G3 has a diameter of some 7.5 nm. It differs from G1 and G2 in sequence, but shares homology with a class of vertebrate hepatic lectins (11). It is present in up to 50% of the particles extracted from mature cartilage (6). A physiological ligand for G3 has not yet been identified although it binds to the same carbohydrate structures as most C-type animal lectins albeit with lower affinity (16).

Cartilage matrix protein (CMP)1 was recently shown to be a compact trimer of identical ellipsoid subunits assembled via their C-terminal extension domains in a coiled-coil α-helix (17, 18). It was first detected as a protein cofractionating with cartilage proteoglycan (19) and later purified from bovine tracheal cartilage under denaturing conditions (20). The molecule consists of three identical subunits of about 50 kDa each which are obtained separately after reduction under denaturing conditions (20). Under native conditions, the trimeric structure of

1 The abbreviations used are: CMP, cartilage matrix protein; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline.
Cmp is retained even upon reduction of the stabilizing interchain disulfide bonds in the C-terminal assembly domain (17, 18).

The primary structures of Cmp deduced from cDNA for chicken (21, 22), man (23), and mouse (24) showed each subunit to consist of two von Willebrand factor A domains, connected via one epidermal growth factor-like domain, and in addition a short C-terminal extension of 43 amino acids. The sequence of Cmp is highly conserved between species as 79% of all amino acid residues are identical between chicken and man. Von Willebrand factor A domains have been implicated in interactions with collagen (for review, see Colombatti and Bonaldo (25)).

Immunolabeling for Cmp was detected on collagen fibrils produced by chondrocytes in culture and Cmp, that had been isolated under denaturing conditions by extraction with 4 M guanidine HCl, was shown to bind to collagens in solid phase assays (26). In the pericellular matrix of cells transfected with Cmp constructs Cmp was also seen in a collagen-independent filamentous network (27).

Cmp is expressed not only in tracheal rings, but also in a limited number of other cartilaginous tissues such as nasal septum, auricular, epiphysial, and xiphisternal cartilage (28, 29). In bovine tracheal cartilage the total amount of Cmp increases greatly with maturation, while the guanidine HCl-soluble pool decreases, indicating an age-dependent cross-linking of Cmp in the matrix (30).

The observed cofractionation of Cmp with aggrecan points to an interaction of these molecules (19). In the present study we describe the isolation of aggrecan-Cmp complexes under conditions minimizing contamination with unrelated proteins. By a combination of biochemical characterization and electron microscopy we demonstrate the age-dependent cross-linking of Cmp to the aggrecan core protein and show distinct binding sites for Cmp on the extended domain D2.

**Experimental Procedures**

**Extraction of Aggrecan-Cmp Complexes—**Fresh bovine tracheal cartilage from steers (fetus, 3–6 months, 18 months, and 6 years) was obtained from the local slaughterhouse. The cartilage was dissected free from surrounding muscle, cut into cubes of about 1 cm³, frozen and stored at −70 °C. An aliquot (10 g) of each sample was thawed in 25 volumes (ml/g wet weight) of prechilled 0.25 M NaCl, 50 mM Tris/HCl, 2 mM phenylmethylsulfonyl fluoride, and 2 mM N-ethylmaleimide, pH 7.4, and homogenized three times for 10 s each using a Polytron homogenizer at full speed. The tissue residue was collected by centrifugation at 17,700 × g for 30 min at 4 °C. This wash cycle was repeated another three times. The prewashed tissue residues were subsequently homogenized in 10 volumes (ml/g wet weight) of 4 M guanidine HCl, 0.01 M Tris/HCl, pH 7.4, in the presence of 2 mM phenylmethylsulfonyl fluoride, 2 mM N-ethylmaleimide, 10 mM EDTA as proteinase inhibitors, and extracted overnight at 4 °C. Alternatively, cartilage was extracted by high speed homogenization for 30 min in either 0.25 M NaCl, 50 mM Tris/HCl, pH 7.4, or in 7 M urea, 0.3 M NaCl, 50 mM Tris/HCl, in the presence of protease inhibitors as described above. Homogenates were centrifuged at 17,700 × g for 30 min at 4 °C and the supernatants recovered.

**Isolation of Aggrecan-Cmp Complexes—**Solid cesium chloride (CsCl) was added to 4 M guanidine HCl extracts to yield a final density of 1.40 g/ml, and the samples were ultracentrifuged at 100,000 × g for 68 h at 18 °C in a 12 × 40-ml fixed angle rotor (type 55.2 Ti, Beckman). The gradient was then divided into five fractions of equal volume from the bottom (D1) to the top (D5). All fractions were analyzed for Cmp by SDS-PAGE and immunoblotting. Further purification steps were performed at 4 °C. D1 fractions were dialyzed against the 4 M guanidine HCl extraction buffer overnight at 4 °C. The supernatant after high speed homogenization was dialyzed against the respective extraction buffers with protease inhibitors and passed through a column (35 × 2.6 cm) of DEAE-Sepharose Fast Flow, which was equilibrated and eluted with the same buffers. Proteoglycans were eluted from the column with the respective extraction buffers, containing 1 M NaCl, and subsequently concentrated by ultrafiltration. Samples (6 ml each) were subsequently applied to a column (90 × 2.6 cm) of Sepharose CL-4B and eluted with 0.5 M Tris/HCl, pH 7.4, at a flow rate of 15 ml/h. Fractions were analyzed by SDS-PAGE, and those corresponding to the void volume pooled and stored at 4 °C.

**Preparation of Antisera—**Cmp was extracted from adult bovine tracheal cartilage using 4 M guanidine HCl and purified as described previously (20). Polyclonal antibodies against Cmp were raised in rabbits, and their specificity was verified as described elsewhere (17, 20). Affinity purification of antibodies was performed on immobilized Cmp immobilized to CNBr-activated Sepharose CL-4B according to the instructions of the manufacturer. The column was washed with TBS, the antibodies were eluted with 3 M KSCN, 50 mM Tris/HCl, pH 7.4, and antibody-containing fractions immediately dialyzed against TBS. These steps were performed in the cold room.

**Conversion of Aggrecan-Cmp Complexes with Chondroitinase Abc and Keratanase—**Aliquots (10 ml) of the void volume pools after Sepharose CL-4B chromatography of proteoglycans prepared from 4 M guanidine HCl, TBS, and urea extracts, respectively, were dialyzed against 0.1 M Tris/HCl, 0.1 M sodium acetate, pH 7.3. They were then digested with chondroitinase ABC (0.0025 unit/mg of proteoglycan) and keratanase (0.05 unit/mg of proteoglycan) for 4 h at 37 °C. Undigested and digested samples were analyzed by SDS-PAGE, and immunoblotting with antibodies against Cmp and the aggrecan G1-domain, respectively. Aliquots (0.5 ml) of the digested samples were applied to a Superox 6 fast protein liquid chromatography-column and eluted with 0.5 M ammonium acetate, pH 7.0, at a flow rate of 0.4 ml/min. Fractions were analyzed by SDS-PAGE and immunoblotting as above, and void volutions, enriched for aggrecan-Cmp complexes, were pooled and finally analyzed by SDS-PAGE and immunoblotting and glycercrol spraying/rotary shadowing electron microscopy. In a separate experiment the distribution of Cmp was quantitated by radioimmunoassay before and after enzyme treatment and correlated to the altered distribution of the core protein in the following way: an aliquot of a D1 fraction of the 6-year-old steer cartilage was subjected to a second CsCl density gradient centrifugation using conditions as described above. The resulting D1D1 bottom proteoglycan fraction of the gradient was subsequently digested with chondroitinase ABC as above and chromatographed on a column (0.8 × 150 cm) of Sepharose CL-6B, eluted with 4 M guanidine HCl, 0.01 M Tris/HCl, pH 7.4. Protein contents of column effluents were determined as the absorbance of 0.280 nm. A sample of each fraction was precipitated with 9 volumes of absolute ethanol before quantitative determination of Cmp by radioimmunoassay as described previously (28).

**Reduction of Aggrecan-Cmp Complexes—**An aliquot of the 6-year-old steer D1D1 fraction was diluted with an equal volume of 8 M guanidine HCl, 0.1 M Tris/HCl, pH 8.0, to achieve a final guanidine HCl concentration of 4 M. Dithioerythritol was added to a concentration of 1 M. The samples were incubated at 25 °C and the sample was incubated for 5 h at 37 °C. The reduced aggrecan-Cmp complexes were subsequently alkylated by addition of iodoacetic acid to a final concentration of 30 mM, followed by incubation overnight in the darkness at room temperature. The sample was diluted to 4 M guanidine HCl by addition of distilled water, followed by chromatography of reduced and alkylated as well as untreated control samples on a column (0.8 × 150 cm) of Sepharose CL-2B, eluted with 4 M guanidine HCl, 0.01 M Tris/HCl, pH 7.4. Column effluents were analyzed for uronic acid content after ethanol precipitation by an automated version (31) of the carbazole reaction (32). Cmp in fractions was determined by radioimmunoassay after ethanol precipitation (28).

**SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting—**SDS-PAGE was performed according to the protocol of Laemmli (33) using gradient gels of 4–15% polyacrylamide. When desired, samples were reduced with 2.5% (v/v) 2-mercaptoethanol in sample buffer. Sample proteins and molecular mass standards were detected by staining with Coomassie Brilliant Blue R or by immunoblotting after electrophoretic transfer to nitrocellulose membranes. Proteins on immunoblots were stained with 10% Ponceau red to use stain that transfer had been adequate. After washing with TBS, the blots were incubated with TBS containing 5% milk powder for 1 h and immunostained by sequential incubations for 1 h at room temperature with the desired rabbit antiserum, diluted 1:200 in TBS with 5% milk powder, and with peroxidase-conjugated swine anti-rabbit IgG (Dako), diluted 1:500 in TBS with 5% milk powder. The blots were developed with 3,3′-diaminobenzidine and bound antibodies were detected using the ECL kit (Amersham Corp.).

**Electron Microscopy—**Aggrecan-Cmp complex samples were dialyzed against 0.2 M ammonium hydrogen carbonate, pH 7.9, overnight at 4 °C. Mixtures of aggrecan-Cmp complexes with affinity purified antibodies to Cmp (typical molar antigen:antibody ratios ranging from 1:5 to 1:1) were treated in the same way. Specimens were prepared for
electron microscopy by glycerol spraying/rotary shadowing (34–36). Briefly, the samples were diluted with an equal volume of 80% glycerol, sprayed onto freshly cleaved mica, dried in vacuo and rotary shadowed with platinum/carbon by means of electron bombardment heating. Evaluation of the data from electron micrographs was done as described previously (37).

RESULTS

Isolation of Aggrecan-CMP Complexes from Tracheal Cartilage—Aggrecan-CMP complexes were isolated from tissues of different ages (fetus, 3–6 months, 18 months, and 6 years) by extraction with 4 M guanidine HCl, followed by CsCl density gradient centrifugation in the same solvent. The distribution of CMP in the different D1–D5 fractions was examined by immunoblotting after SDS-PAGE and showed an age-dependent pat-
tern (Fig. 1). In fetal tracheal cartilage, CMP was predominant in the top fraction (D5). With increasing age the distribution of CMP shifted toward the high buoyant density bottom proteoglycan fraction (D1). The 6-year-old cartilage extract contained the highest amounts of CMP in D1, but also in the D2–D4 fractions, indicating the presence of complexes of CMP with intact and fragmented aggrecan throughout the CsCl gradient.

CMP was partially separated from aggrecan in SDS-PAGE under reducing conditions (Fig. 1).

The aggrecan-CMP containing D1 fractions from 3–6-month and 6-year-old cartilage were selected for further purification by chromatography on Sepharose CL-4B (elucent 4 M guanidine HCl-containing buffer) before (a) and after (b) reduction and alkylation. The curves designate aggrecan as monitored by the absorbance at 530 nm (—) after carbazole assay (arbitrary units, right side) and CMP (-----) as determined by radioimmunoassay (µg/ml, left side). Note the shift of about two thirds of CMP toward the total volume in b, indicating a dissociation and partial release of CMP subunits after reduction.

In attempts to exclude the possibility of artefactual, non-

physiological interactions between CMP and aggrecan, induced by guanidine HCl or CsCl, we performed parallel control experiments. We isolated aggrecan-CMP complexes under native conditions by high speed homogenization of 6-year-old bovine tracheal cartilage in 0.25 M NaCl, 0.05 M Tris/HCl, pH 7.4. Alternatively, aggrecan-CMP complexes were isolated by high speed homogenization of samples of the same tissue in buffer containing 7 M urea. In order to avoid high concentrations of CsCl, aggrecan-CMP complexes were purified by ion exchange chromatography on DEAE-Sepharose and gel filtration on Sepharose CL-4B instead of isopycnic density gradient centrifugation. Analysis of such aggrecan-CMP complexes by immunoblotting after SDS-PAGE and electron microscopy showed the same distribution of CMP. We distinguished a first pool comprising soluble CMP, a second pool consisting of CMP associated with aggrecan, but not covalently bound to it, and a third pool apparently being covalently cross-linked to aggrecan and partially released by reduction. Furthermore, electron microscopical inspection demonstrated aggrecan-CMP complexes similar to those after guanidine HCl extraction and CsCl den-
sity gradient centrifugation (results not shown). We therefore conclude that the observed covalent association of CMP with the aggrecan core protein reflects the in situ situation.

**Enzymatic Deglycosylation of Aggrecan-CMP Complexes**—Void volume fractions from Sepharose CL-4B were subsequently digested with chondroitinase ABC and keratanase. They were analyzed by nonreducing SDS-PAGE, followed by immunoblotting with antibodies to CMP and the aggrecan G1 domain. Comparison of the different staining patterns revealed the presence of CMP in all the aggrecan containing samples, even after enzyme treatment to remove the glycosaminoglycan side chains (Fig. 3). This shows that CMP is bound to the core protein rather than being linked to the glycosaminoglycan side chains. In the same experiment, an age-dependent net increase in CMP cross-linked to the aggrecan core protein was observed. The respective sample from immature cartilage showed only little staining for CMP, when compared with the sample from old cartilage, while the staining pattern obtained for aggrecan G1 domain was similar to that of old cartilage (Fig. 3).

In a parallel analytical experiment, a D1 sample from 6-year-old cartilage was used to further characterize the interaction of CMP with aggrecan core protein. The respective sample was subjected to a second CsCl density gradient centrifugation in buffer containing 4 M guanidine HCl, thus minimizing contamination of the preparation with unbound CMP. An aliquot of the D1D1 fraction was chromatographed on Sepharose CL-6B in buffer containing 4 M guanidine HCl after digestion with chondroitinase ABC. The distribution of CMP in column effluents was quantitated by radioimmunoassay and correlated to the altered distribution of the core protein upon deglycosylation (Fig. 4). The CMP coeluted with a distinct subpopulation of the core protein and was distinctly shifted from the void volume position of nondigested material (data not shown). The results provide strong evidence for a CMP-core protein interaction rather than a binding of CMP to the glycosaminoglycan side chains.

**Reduction of Aggrecan-CMP Complexes**—Chondroitinase and keratanase digested purified aggrecan-CMP complexes, as described above, were subjected to SDS-PAGE under reducing conditions. They were subsequently analyzed by immunoblotting with antibodies to CMP and the aggrecan G1 domain. A partial release of CMP subunits from the aggrecan core protein was observed, as free CMP subunits appeared upon reduction (Fig. 5b). This observation was examined in more detail using aggrecan-CMP complexes in D1D1 fractions, as above. Samples were chromatographed on Sepharose CL-2B in buffer containing 4 M guanidine HCl before and after reduction and alkylation. Quantitation of CMP by radioimmunoassay showed release of 2/3 of the CMP upon reduction (Fig. 5b), whereas one third of the total CMP still coeluted with aggrecan (Fig. 5a). Taken together, these findings indicate a nonreducible covalent cross-linking of CMP to the aggrecan core protein via one of its three subunits, where the other two subunits can be released by reduction of the interchain disulfide bonds under dissociative conditions.

**Electron Microscopy of Aggrecan-CMP Complexes**—Fractions enriched in aggrecan-CMP complexes from tracheal cartilage of different ages were visualized by electron microscopy after glycerol spraying/rotary shadowing. Individual CMP molecules were observed as globular particles, found at distinct locations on the extended core protein domain E2 (Figs. 6a and 7, a and c). Labeling of aggrecan-CMP complexes with affinity purified antibodies to CMP confirmed the identity of these particles
TABLE I

| Extract | Cartilage |
|---------|-----------|
| 3-6 Months | 6 Years |
| Globular particles on E2, % of total core proteins | 21 | 38 |
| Immunostaining with anti-CMP, % of total core proteins | 14 | 65 |
| Percent of total core proteins carrying | |
| One molecule of CMP | 19 | 26 |
| Two molecules of CMP | 2 | 10 |
| Three molecules of CMP | 0 | 2 |

respectively, and two minor binding sites at 80 nm in young tissue, the occupation of the binding sites already seen in young tissue, CMP with aging appears to be due to an increasing frequency of occupation of the different binding sites for CMP on E2 varied with aging. Furthermore, electron microscopic examination revealed the extended aggrecan core protein domain E2. The relative extent of occupation of the different binding sites varies with aging. Electron microscopy revealed the appearance of additional binding sites on E2 for CMP and an increased decoration of E2 with CMP upon aging. This is in general accordance with the observation of an overall age-dependent increase in total tissue CMP is accompanied by a shift from CMP in the first pool to the third, tightly bound, pool.

Electron microscopy revealed distinct binding sites of CMP on the extended aggrecan core protein domain E2. The relative extent of occupation of the different binding sites varies with aging. Furthermore, electron microscopic examination revealed the additional appearance of new binding sites on E2 for CMP and an increased decoration of E2 with CMP upon aging. This is in general accordance with the observation of an overall age-dependent increase in apparent covalently bound CMP, as shown by biochemical analysis of the fractions enriched in aggrecan-CMP complexes and from previous results showing accumulation of CMP within tracheal cartilage with age (30). The periodic appearance of CMP binding sites along E2 may be due to repetitive sequences in the core protein which could contain motifs for CMP binding. Alternatively, this might reflect less glycosylated regions of E2 which are thus accessible to CMP binding and subsequent cross-linking. The age-dependent altered occupation of the different binding sites with CMP may reflect a change in the availability of a given binding site for CMP in situ.

The mechanism for covalent cross-linking of CMP is unknown. A possible candidate enzyme for cross-linking in situ, tissue transglutaminase, is expressed in some types of cartilage, including tracheal rings (38). However, in vitro assays using purified tissue transglutaminase, showed no incorporation of radiolabeled putrescine into CMP, aggrecan or the aggrecan core protein (results not shown).

The interaction of CMP with collagen fibrils has been postulated by Winterbottom et al. (26). This work was based on immunolabeling for CMP of collagen fibrils in the pericellular matrix of cultured chondrocytes, and binding of collagen to 4 M guanidine HCl-extracted CMP in enzyme-linked immunosorbent style assays. If this interaction truly occurs in vivo, and the interaction with aggrecan core protein shown in the present study exists simultaneously, CMP might play an integrating role in cartilage extracellular matrix organization as a bridging molecule between the two major cartilage matrix constituents, aggrecan and collagen.

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