Cartilage and tendon extracellular matrices are composed of collagens, proteoglycans, and a number of non-collagenous proteins. Cartilage oligomeric matrix protein (COMP) is a prominent such protein, structurally related to the thrombospondins. We found that native COMP binds to collagen I/II and procollagen I/II and that the interaction is dependent on the divalent cations Zn$^{2+}$ or Ni$^{2+}$, whereas Ca$^{2+}$, Mg$^{2+}$, and Mn$^{2+}$ did not promote binding. Using a solid phase assay, Scatchard analysis identified one class of binding site with a dissociation constant ($K_d$) close to 1.5 nM in the presence of Zn$^{2+}$. The results were confirmed by studies using surface plasmon resonance. Furthermore, metal chelate chromatography demonstrated that COMP bound Zn$^{2+}$ and Ni$^{2+}$. Electron microscopy showed that the interaction occurred at four defined sites on the 300-nm collagen and procollagen molecules. Two were located close to each end, and two at 126 and 206 nm, respectively, from the C-terminal. COMP interacted via its C-terminal globular domain and significantly only in the presence of Zn$^{2+}$.

The major structural constituents of the ECM in cartilage are proteoglycans and collagens. One of the more prominent non-collagenous proteins is COMP. This protein was initially found in articular, nasal, and tracheal cartilage (1), but has later been isolated from tendon (2, 3), where also the corresponding mRNA was demonstrated (3). In the growth plate, COMP is primarily observed in the proliferative region, where it is prominent pericellularly (4, 5), indicating a role in cell growth and matrix development. In more developed articular cartilage, COMP is a major non-collagenous matrix component, primarily located interterritorially, especially in the more superficial part of the tissue. This high expression of COMP in mature articular cartilage may be induced by the high mechanical load on the tissue. In support, a non-weight-bearing equine tendon shows considerably lower COMP levels than the contralateral weight-bearing one (3).

COMP was first isolated from bovine articular cartilage by extraction under denaturing conditions with 4 M guanidine HCl (1). Native COMP has been isolated from Swarm rat chondrosarcoma (6), bovine cartilage (7), and human articular cartilage (8) under mild non-denaturing conditions by extraction with 10 mM EDTA, indicating that the interaction of COMP with components of the ECM depends on divalent cations. Structurally, COMP is related to the thrombospondins (9), having the same molecular domain arrangement of a series of four type 2 (epidermal growth factor) repeat domains followed by seven type 3 domains (calcium binding). COMP, however, is a pentameric glycoprotein consisting of identical 86650 Da subunits, each substituted with two N-linked oligosaccharides (10). The five monomers are joined by interactions between their N-terminal portions that form a cylindrical structure (6). The interactions involve the formation of a five-stranded coiled coil (11) from an $\alpha$-helical domain at the N terminus and are stabilized by two disulfide bonds. This structure shows a remarkable similarity to proposed models for trans-membrane ion-channels (12), although the role for such a protein in the ECM remains an enigma. The $\alpha$-helical oligomerization domain is followed by a flexible strand and, at the C-terminal, a globular domain (6) that shows homology to the thrombospondins (9). It is possible that the globular C termini form multimeric binding sites for the interaction with other ECM components.

Bovine (9) and human (13) COMP contain an RGD sequence although at different locations, indicating that COMP may mediate cell binding (7). Rat COMP, however, does not contain any RGD sequence (9), and the primarily interterritorial localization of the protein in adult human cartilage (9) suggests that the promotion of cell binding is not a primary function of COMP. It is possible that the adhesive properties of COMP are indirect, mediated by an ECM interaction partner. In line, no cell surface receptor for COMP has been isolated. That COMP may have important functions in the ECM is illustrated by the fact that a mutation in the potentially Ca-binding domain has been shown to lead to impaired secretion and severe multiple epiphyseal dysplasia (14).

Collagens constitute a large family of molecules with some 20 different members. In cartilage and tendon collagens II and I, respectively, are major components. Collagens are rodlike molecules, composed of three $\alpha$-chains forming a very stable triple-helical structure. Each polypeptide chain is largely made up of a repeating sequence of -Gly-X-Y-, where X often is proline and Y hydroxyproline (15). In collagens I and II, many such monomers are assembled in a very specific fashion into fibrils, which are stabilized by intra- and intermolecular cross-links (16). Obviously, the organization of collagen fibrils in the ECM is of fundamental importance to the structure and function of e.g. cartilage and tendon. In the tissue the collagen fibrils associate

---

*This work was supported by grants from Swedish Medical Research Council, the Royal Physiographic Society, Greta and Johan Kock’s Foundation, Anna-Greta Crafoord’s Foundation for Research on Rheumatology, Österlund’s Foundation, Kung Gustav V’s 80-års Foundation, and the Medical Faculty of the University of Lund. 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.

† To whom correspondence should be addressed: Lund University, Department of Cell and Molecular Biology, Section for Connective Tissue Biology, P. O. Box 94, S-221 00 Lund, Sweden. Tel.: 46 46 222 8571; Fax: 46 46 211 3417; E-mail: Dick.Heinegard@medkm.lu.se.

‡ The abbreviations used are: ECM, extracellular matrix; COMP, cartilage oligomeric matrix protein; PAGE, polyacrylamide gel electrophoresis.

1 The abbreviations used are: ECM, extracellular matrix; COMP, cartilage oligomeric matrix protein; PAGE, polyacrylamide gel electrophoresis.

2 P. Lorenzo and D. Heinegård, unpublished results.

---

© 1998 by The American Society for Biochemistry and Molecular Biology, Inc.

Printed in U.S.A.

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 273, No. 32, Issue of August 7, pp. 20397–20403, 1998

20397

This paper is available on line at http://www.jbc.org
to form fibers, which, in turn, form a network that is stabilized by cross bridging molecules. These include other collagens, e.g., collagen IX in cartilage (17) as well as noncollagenous molecules such as decorin (18–21), fibromodulin (19, 22, 23) and lumican (24), all capable of specific binding to triple helical collagen. In this report we present new data showing that native COMP is a novel interaction partner for collagens and that the interaction requires specific divalent cations.

EXPERIMENTAL PROCEDURES

Isolation of COMP

Extractions—Fresh adult bovine fetlock joint articular cartilage (120 g) obtained from the local slaughterhouse was homogenized on ice with a Polytron homogenizer in 10 volumes (ml/g) of preextraction buffer (phosphate-buffered saline containing proteinase inhibitor mix: 0.10 mM e-aminoacproic acid, 5 mM benzamidine hydrochloride, and 10 mM N-ethylmaleimide). The homogenate was preextracted for 1 h at 4 °C and centrifuged at 20,000 rpm (Beckman, JA 20 rotor) for 30 min at 4 °C. The pellet was resuspended in 10 volumes of the same buffer by brief homogenization, washed for 15 min at 4 °C and centrifuged as above. COMP was extracted from the residue with 10 volumes (ml/g) of extraction buffer (preextraction buffer now containing 10 mM EDTA) over-night at 4 °C. The residue was reextracted for 15 min with 10 volumes (ml/g) of extraction buffer. Remaining proteins were extracted with 4 mM guanidine HCl, 50 mM sodium acetate, 10 mM EDTA, pH 5.8, containing protease inhibitor mix. Aliquots of 50 μl from all extracts were analyzed for COMP by 4–16% SDS-PAGE (25) and Western blotting (26).

Metal Chelate Affinity Chromatography

COMP (40 mg/ml of 1 ng/mm2) (29). The binding assay was initially optimized by varying the concentration of collagen II (0.18 to 3 μM, flow 5 μl/min, sample volume 20 μl), the flow (5–80 μl/min, concentration 0.38 μM, sample volume 40 μl) and the sample volume (50–250 μl, flow 80 μl/min, concentration 0.38 μM); in total, 15 different variables. Proteins remaining on the surface were eluted with 5 mM EDTA. In the first binding experiment, 12.5 μg/ml collagen II was captured on the COMP and blank surfaces in TBS containing 1 mM ZnCl₂ or 1 mM CaCl₂ at a flow rate of 80 μl/min and a sample volume of 160 μl. In a second experiment 12.5 μg/ml COMP or, as a negative control, 10 μg/ml of ovalbumin were pumped over the collagen II and blank surfaces in TBS with 1 mM ZnCl₂ at a flow of 20 μl/min and a volume of 20 μl. The BIA evaluation software version 2.1 were used to calculate the Kᵢ (the binding (30)).

RESULTS

Electron Microscopy

COMP-procollagen samples (about 50 μg/ml at a molar COMP:procollagen ratio of 1:8) and COMP-collagen samples (about 200 μg/ml at a molar COMP:collagen ratio of 1:2) were dialyzed overnight at 4 °C against 0.2 M ammonium formate, pH 7.4, containing 1 mM ZnCl₂ or 1 mM EDTA. Glycerol spraying and rotary shadowing for electron microscopy were performed as described previously (31–33). Briefly, the samples were diluted to a final concentration of 10 μg/ml with appropriate glycerol (52%) and 40% freshly cleaned mica disks, dried in vacuo, and rotary shadowed with platinum/carbon by means of electron bombardment heating. Alternatively, complexes were subjected to negative staining prior to electron microscopy. Samples of the respective proteins were incubated in the dialysis buffer described above and allowed to react for 2 h at 4 °C. The samples were diluted to a final concentration of 2 μg/ml with 50 mM Tris-Cl, 0.15 mM NaCl, pH 7.4. They were then adsorbed onto carbon-coated grids for 5 min, washed with one drop of water, and stained with 0.75% uranyl formate. The grids were rendered hydrophilic by glow discharge in air. Samples were observed in a Jeol 1200 EX electron microscope operated at 60 kV accelerating voltage. Evaluation of the data from electron micrographs was done as described previously (34).

Isolation of COMP—Bovine articular cartilage was sequentially extracted first with phosphate-buffered saline containing protease inhibitors and N-ethylmaleimide, followed by this buffer also containing 10 mM EDTA (6). To estimate the size of calculated molecular mass for COMP pentamer of 433,250 Da (10). Bound COMP was detected using polyclonal rabbit anti-bovine COMP (1) followed by alkaline phosphatase-conjugated swine anti-rabbit IgG using p-nitrophenyl phosphate as the chromogenic substrate and recording the absorbance at 405 nm.

Surface Plasmon Resonance Assay

The BIAcore™ 2000 system (BIAcore AB, Sweden) was used to characterize the interaction and determine the Kᵢ between COMP and collagen II. Pure COMP and collagen II, respectively, were immobilized at 25 °C at a flow of 5 μl/min. Briefly, a CM5 sensor chip (BIAcore AB, Uppsala, Sweden) with a carboxymethylated dextran surface was activated with 40 μl of a mixture of 50 mM N-hydroxysuccinimide and 200 mM N-ethyl-N′-dimethylaminopropylcarbodiimide. COMP (40 μl of 50 μg/ml in 10 mM sodium citrate, 0.15 mM NaCl, pH 5.2 or collagen II (40 μl of 100 μg/ml) in 10 mM sodium citrate, pH 5.0, were immobilized. One surface was used as blank containing no coupled protein. Remaining activated complexes were blocked with 40 μl of 1 M ethanolamine in 0.1 M NaHCO₃, 0.5 mM NaCl, pH 8.3. The immobilization resulted in 840 resonance units for COMP and 3000 resonance units for collagen II. After rinsing as above, the collagen-coated wells were incubated for 1 h at 4 °C and centrifuged at 1000–400 mM linear NaCl gradient in the same buffer. Fractions of 1 ml were collected, and aliquots of 50–250 μl of each 1-ml fraction were analyzed for COMP by SDS-PAGE (25) and Western blotting (26). Protein concentrations were determined using the Coomassie Plus reagent (Pierce).
the EDTA-extractable pool of COMP, the tissue residue was further extracted with guanidine HCl (see “Experimental Procedures”) to release remaining proteins. Some 40% of the total amount of COMP present in the tissue was recovered in the EDTA extract (not shown) as judged by the intensity of the bands upon SDS-PAGE (25) of the different extracts. The EDTA extract was fractionated by chromatography on Fast Flow DEAE-Sepharose, heparin-Sepharose, and MonoQ columns. The typical yield of native, EDTA-extracted COMP was approximately 0.2 mg/g of cartilage tissue (wet weight), and the final product appeared pure, without detectable non-COMP components as confirmed by SDS-PAGE (Fig. 1) and Western blotting (not shown).

**Interaction between COMP and Collagens in a Solid Phase Assay**—Binding of COMP to collagens coated onto microtiter plates was demonstrated using an enzyme-linked immunoabsorbent assay procedure. To determine the optimal coating density, different concentrations of collagens I and II were adsorbed to the plate followed by addition of 5 μg/ml COMP in the presence of 1 mM Zn$^{2+}$. The binding became saturated at a coating density of 1 μg/ml for the collagens (not shown), which was used in some of the subsequent experiments. The binding of COMP to ovalbumin, used to block unoccupied sites on the plastic surface, was negligible.

**Effect of Divalent Cations**—In the nominal absence of divalent cations (1 mM EDTA) the binding that is seen in the presence of 1 mM Zn$^{2+}$ was reduced to control levels (ovalbumin). Substitution of Zn$^{2+}$ for Ca$^{2+}$, Mg$^{2+}$, or Mn$^{2+}$ also reduced binding to control levels. Among the divalent cations tested, the interaction between COMP and collagens I and II displayed a preference for Zn$^{2+}$ (Fig. 2, A and B), and the binding was saturated at 0.5 mM of Zn$^{2+}$. Also Ni$^{2+}$ promoted binding between COMP and these collagens, however less efficiently (70% of the binding obtained with Zn$^{2+}$). To determine whether calcium ions had an inhibitory effect on the Zn$^{2+}$-supported binding between COMP and collagens I and II, different concentrations of Ca$^{2+}$ were combined with a constant concentration of 0.5 mM zinc. The binding was inhibited by approximately 40% at a calcium concentration of 1 mM but was not further inhibited at up to 5 times higher concentrations of calcium over zinc (Fig. 3).

**Dissociation Constants**—To assess the strength of the interaction between COMP and collagens I and II the COMP concentration was varied in the presence of 0.5 mM Zn$^{2+}$ and a coating density of 20 μg/ml of the respective collagen. Under these conditions the binding displayed saturation at approximately 15 nM COMP both for collagen II and for collagen I (Fig. 4A and B). Scatchard analysis (35) of the data, assuming one class of binding site, demonstrated $K_d$ values of 1.51 ± 0.25 nM for collagen I (Fig. 4A) and 1.72 ± 0.16 nM for collagen II (Fig. 4B).

**Interaction between COMP and Collagen II Studied by Surface Plasmon Resonance**—This system was first optimized for COMP bound to the surface by adding collagen II in the presence of Zn$^{2+}$. The calculated mean $K_d$ value found for the 15 different experimental settings was 3.0 ± 1.5 nM. Further characterization of the interaction at the optimum binding conditions in the presence of Zn$^{2+}$ or Ca$^{2+}$ showed that no binding occurred between COMP and collagen II in the presence of Ca$^{2+}$ (Fig. 5). The COMP-collagen II interaction could be demonstrated also in the reverse manner, i.e. by passing COMP over a collagen II surface in the presence of Zn$^{2+}$, where ovalbumin, the negative control, did not bind (Fig. 5, inset).

**COMP’s Affinity for Divalent Cations**—To determine whether COMP had direct affinity for divalent metal ions, the protein was passed over metal chelate columns charged with Zn$^{2+}$, Ca$^{2+}$, Ca$^{2+}$, Cd$^{2+}$, Mg$^{2+}$, Mn$^{2+}$, and Ni$^{2+}$. When applied to an uncharged column or to columns charged with Cd$^{2+}$, Mg$^{2+}$, or Mn$^{2+}$, COMP did not bind and was recovered in the flow-through.
COMP was retained on columns charged with Zn\(^{2+}\), and more than 50% of the applied protein was eluted with 50 mM EDTA (Fig. 6). COMP was retained also on a Ni\(^{2+}\) column. In this case, it did not elute with 50 mM EDTA, but elution required 7% acetic acid.

Electron Microscopy—Complexes between COMP and collagen were visualized by electron microscopy after spraying from glycerol/buffer mixtures and rotary shadowing (Fig. 7, A and B) as well as after adsorption to carbon films and negative staining (Fig. 7 C). Both techniques produced images where the pentameric COMP structure, with its five globular C-terminal globules, and individual 300 nm collagen monomers could be clearly distinguished. Rotary shadowing images revealed COMP-collagen I/II complexes (Fig. 7 A) and COMP-procollagen I/II complexes (Fig. 7 B), respectively. A key observation was that only the C-terminal globule of COMP interacted with the collagen monomer. This was further underlined by negative staining of COMP-collagen I/II complexes, where the N-terminal oligomerization domain of COMP appeared more distinct and clearly distinguished from collagen filaments (Fig. 7C). By using procollagen it was possible to establish the polarity of the monomer, as the C-terminal propeptide adopted a globular conformation that was clearly visible in the micrographs (Fig. 7B). Statistical analysis of the binding of COMP to procollagen I/II in the presence of zinc revealed four binding sites situated at 0 (the C-terminal), 126, 206, and 300 nm (the N-terminal) (Fig. 8, A and B). The COMP molecules were roughly equally

**FIG. 3.** COMP binding to collagen I and II is affected by different ratios of Zn\(^{2+}\):Ca\(^{2+}\) in solid phase assay. Collagens I (■—■) and II (○—○), respectively, were coated 1 \(\mu\)g/ml in 0.1 m acetic acid, pH 2.5, on a 96-well polystyrene plate over night and incubated for 1 h with 5 \(\mu\)g/ml COMP in 25 mM Tris-HCl, 0.15 m NaCl, 0.5 mM ZnCl\(_2\), pH 7.4, also containing 0.01–2.56 mM CaCl\(_2\). Bound COMP was detected using a polyclonal rabbit anti-bovine COMP followed by alkaline phosphate-conjugated swine anti-rabbit IgG using p-nitrophenyl phosphate as the chromogenic substrate and recording absorbance at 405 nm. Data are means of quadruplicate determinations ± S.D.

**FIG. 4.** COMP binding to collagen I and II in the presence of Zn\(^{2+}\) and their dissociation constants in solid phase assay. Collagens I (A, ■—■) and II (B, ○—○), respectively, were coated at 20.0 \(\mu\)g/ml in 0.1 m acetic acid, pH 2.5, on a 96-well polystyrene plate overnight and incubated with 0.37–190 nM COMP in 25 mM Tris-HCl, 0.15 m NaCl, 0.5 mM ZnCl\(_2\), pH 7.4, for 1 h to obtain binding saturation curves. Bound COMP was detected using a polyclonal rabbit antiboine COMP followed by alkaline phosphate-conjugated swine anti-rabbit IgG using p-nitrophenyl phosphate as the chromogenic substrate and recording absorbance at 405 nm. Inset, the values were analyzed by Scatchard plots to calculate the dissociation constant between COMP and collagen I and II. Data are means of quadruplicate determinations ± S.D.

**FIG. 5.** COMP binding to collagen II detected by surface plasmon resonance assay. COMP immobilized on a CM5 sensor chip was allowed to interact with 12.5 \(\mu\)g/ml collagen II in 25 mM Tris-HCl, 0.15 m NaCl, pH 7.4, in the presence of 1 mM CaCl\(_2\) (----), or in the presence of 1 mM ZnCl\(_2\) (- - - - - - ), injected with a flow of 80 \(\mu\)l/min and a volume of 160 \(\mu\)l. Inset, collagen II immobilized on a CM5 sensor chip interacts with 12.5 \(\mu\)g/ml COMP in 25 mM Tris-HCl, 0.15 m NaCl, 1 mM ZnCl\(_2\), pH 7.4 (----), but not with 10 \(\mu\)g/ml ovalbumin (- - - - - - ), injected with a flow of 20 \(\mu\)l/min and a volume of 20 \(\mu\)l.

**FIG. 6.** COMP binding to zinc upon zinc chelate affinity chromatography. COMP in 25 mM Tris, 0.5 m NaCl, pH 7.4 (TBS) was recirculated through a HiTrap column charged with zinc for 20 h. COMP was identified in column effluent fractions (■—■) by SDS-PAGE 4–16% gradient gel electrophoresis, and the bands were quantified by measuring the optical density (Gel Pro Analyzer). Fraction 1, flow-through; fractions 2–5, washed with TBS; fractions 6–11, COMP eluted with TBS with 50 mM EDTA, and fractions 12–15, 7% acetic acid.

Electron Microscopy—Complexes between COMP and collagens were visualized by electron microscopy after spraying from glycerol/buffer mixtures and rotary shadowing (Fig. 7, A and B) as well as after adsorption to carbon films and negative staining (Fig. 7C). Both techniques produced images where the pentameric COMP structure, with its five globular C-terminal globules, and individual 300 nm collagen monomers could be clearly distinguished. Rotary shadowing images revealed COMP-collagen I/II complexes (Fig. 7A) and COMP-procollagen I/II complexes (Fig. 7B), respectively. A key observation was that only the C-terminal globule of COMP interacted with the collagen monomer. This was further underlined by negative staining of COMP-collagen I/II complexes, where the N-terminal oligomerization domain of COMP appeared more distinct and clearly distinguished from collagen filaments (Fig. 7C). By using procollagen it was possible to establish the polarity of the monomer, as the C-terminal propeptide adopted a globular conformation that was clearly visible in the micrographs (Fig. 7B). Statistical analysis of the binding of COMP to procollagen I/II in the presence of zinc revealed four binding sites situated at 0 (the C-terminal), 126, 206, and 300 nm (the N-terminal) (Fig. 8, A and B). The COMP molecules were roughly equally
distributed between these sites, possibly with a slight preference for the C-terminal of procollagen I/II. In the presence of EDTA, i.e. in the nominal absence of Zn$^{2+}$, the four sites still showed, distinguishable, although much reduced, preferential binding. However, considerable randomized binding occurred between the sites (Fig. 8, C and D), and the total number of collagen molecules containing bound COMP was reduced from 60% to 10%. The same analysis when applied to COMP-collagen I/II complexes yielded three apparent binding sites: at 0 nm, 94 nm and 126 nm (Fig. 9, A and B). This is consistent with the results obtained for procollagen, considering that it is not possible to determine the polarity of the collagen monomer. Again, substitution of Zn$^{2+}$ for EDTA resulted in reduced relative binding at the specific binding sites and increased random binding (Fig. 9, C and D).

**DISCUSSION**

In the present study we demonstrate that COMP, abundant in cartilage and tendon extracellular matrix, is a novel interaction partner for the fibril forming collagens I and II. This suggests that one function for COMP is to influence the organization of collagen fibrils, thereby contributing to tissue structure and function.

In preliminary experiments aiming at identifying ECM-components interacting with COMP, sulfate-labeled glycosaminoglycans from cultured bovine chondrocytes and Swarm rat
chondrosarcoma cells were passed over COMP affinity columns. Persistently, proteoglycans, including aggrecan, biglycan, and fibromodulin, were bound to the column. However, using purified proteoglycans no COMP-proteoglycan interaction could demonstrate suggesting that the interaction was mediated by other components. The previously described interactions between small proteoglycans and collagens (19) prompted us to test for a direct binding between collagens and COMP. Indeed, such an interaction could be demonstrated and was shown to depend on the presence of divalent cations.

The observation that there is a large pool of COMP in cartilage that is readily extractable by physiological buffers containing EDTA (6), indicated that the anchorage of COMP in the ECM depends on divalent cations. This interpretation is corroborated by the present data that the binding of COMP to collagens I and II in a solid phase assay is promoted by Zn$^{2+}$. On the other hand, the divalent cations Ca$^{2+}$, Mg$^{2+}$, and Mn$^{2+}$ did not induce binding, and, if showing any effect, rather inhibited the weak residual binding between the proteins observed in the nominal absence of divalent metal ions (Fig. 2, A and B). Moreover, Ca$^{2+}$ at a concentration of 2 mM did, although only partly, inhibit the COMP-collagen interaction induced by 0.5 mM Zn$^{2+}$ (Fig. 3). Binding was not detected between COMP and collagen II in the presence of Ca$^{2+}$ with no Zn$^{2+}$, in studies using surface plasmon resonance (Fig. 5).

Further characterization of the COMP-collagen interaction by Scatchard analysis was consistent with only one class of binding sites (Fig. 4, insets). The apparent dissociation constants were 1.51 ± 0.25 nM and 1.72 ± 0.16 nM for collagen I and II, respectively. The dissociation constant of 3.0 ± 1.5 nM found upon surface plasmon resonance analysis of the interaction between COMP and collagen II was comparable to the data from the solid phase assay. Clearly, the high affinity of the interaction strongly suggests that COMP and collagen may interact also in vivo, where they are found in the same interterritorial compartment of e.g. adult articular cartilage (4).

Metal chelate affinity chromatography revealed that COMP binds Zn$^{2+}$ (Fig. 5) and Ni$^{2+}$. Columns charged with Ca$^{2+}$, Mg$^{2+}$, and Mn$^{2+}$ did not retain COMP (not shown), but as these ions are poorly chelated by the currently available chelating matrices the interpretation of the results is ambiguous. As a matter of fact, the observation that Ca$^{2+}$ partly inhibited the binding induced by Zn$^{2+}$, suggests that COMP can also bind Ca$^{2+}$. This would be expected as the COMP monomer contains several putative Ca-binding epidermal growth factor-domains and calcium binding thrombospondin type 3 repeats (9). Two of the epidermal growth factor domains have amino acid sequences typical for calcium binding (36, 37). The thrombospondin type 3 repeats are rich in Asp and Asn, just like cadherins that are known calcium-binding proteins (38). Added to this, Cys and His are very common throughout the molecule, and these residues are known to coordinate divalent ions (39). Further indication that Zn$^{2+}$ promotes the interaction by binding to COMP, rather than to collagen, is that the previously described interaction between fibromodulin and collagen I or II was totally unaffected by the presence of Zn$^{2+}$ (not shown). As this interaction depends on the native structure of collagen (21), it is not likely that Zn$^{2+}$ induces significant change in the collagen. We therefore suggest that the role of Zn$^{2+}$ is to induce a conformation of COMP that promotes binding to collagen. The observation that Ca$^{2+}$ only partially competes with the Zn$^{2+}$-induced binding (Fig. 3) indicates that the two ions bind to different sites.

Visualization of the COMP-collagen complexes by electron microscopy clearly showed that it is the C-terminal globular part of COMP that mediates the interaction. Furthermore, statistical evaluation of the binding demonstrated the presence of four distinct binding sites on collagen (Fig. 7, A–C). The COMP molecules distributed rather equally between these sites, indicating their similar or equal affinities for COMP. This was corroborated by the Scatchard plot data and the surface plasmon resonance data indicating one type of strong binding. The use of procollagen made it possible to determine the polarity of the collagen monomer as the C-terminal propeptide appeared as a globule in the micrographs (Fig. 7B). With pepsin-extracted collagens, not containing the propeptides, the N and C termini could no longer be distinguished. Consequently, the four binding sites identified at 0 (C terminus), 126, 206, and 300 nm (N terminus) on procollagen were reduced to only three sites on collagen; one including the N and C termini at an end (Fig. 9, A and B). The fact that binding was observed at the ends of the filamentous part of procollagen as well as of pepsinized collagen can be taken to indicate that also these active sites are in the triple helical domain, since it is likely that the structures corresponding to the short telopeptides have quite a different conformation in procollagen. Another observation was that the binding at 126 nm from the C-terminal is located very near the fibronectin binding site, amino acids 757–791 (40), as well as the collagenase cleavage site, amino acids 775–776 (41). Some COMP-collagen complexes were still observed in the presence of EDTA, but the portion of COMP molecules engaged in specific binding was much reduced. In addition, the distribution of COMP along the collagen monomer became more randomized, although there was some preference for the four sites (Fig. 9, C and D). This residual binding, also observed in the solid phase assay, may be due to some Zn$^{2+}$ or Ni$^{2+}$, remaining bound to COMP even in the presence of EDTA, and, if so, indicates a very tight coordination of these ions by COMP.

The structurally related thrombospondin type 1 has similarly been shown to bind to collagens I–V at one end of the pepsinized, triple helical collagen, but in the presence of Ca$^{2+}$ or EGTA (42, 43). It has also been shown that thrombospondin type 1 binds to Ca$^{2+}$ (44) and that this binding can be inhibited to 40% by Zn$^{2+}$ (45).

An important issue is whether these observations are physiologically relevant and one consideration is then the concentrations and relative abundance of divalent ions in cartilage. The concentrations of the divalent ions have been measured by different techniques, giving somewhat different results. In summary, concentrations of zinc ranging from 0.16 to 1.85 mM have been determined (46, 47) and calcium values reported range from 5 to 98 mM (47–51). It has been reported that the mass ratio between Ca$^{2+}$ and Zn$^{2+}$ is 5:1 in resting cartilage of the growth plate (52), but in articular cartilage the proportion is 50:1 (47). However, little is known about the distribution in the different layers and compartments in cartilage. It has been demonstrated that the proportion of divalent cations vary between different layers in epiphyseal growth cartilage (49), scapular growth cartilage (52), and in adult articular cartilage (47). This distribution is likely to be influenced by the molecular composition of the ECM. The overall concentrations of zinc shown for articular cartilage is well above the concentration critical for optimal COMP binding. On the other hand if the overall ratio of calcium to zinc is as high as around 50 (47), it is possible that local variations in this ratio may modulate COMP-collagen interactions. The notion that Zn$^{2+}$ and Ni$^{2+}$ may play important biological roles in cartilage organization is supported by the observation that these ion species appear to modulate the biochemical and functional properties of link protein in bovine articular cartilage (53).

COMP is an enigmatic protein that despite its high expression in cartilage has not been assigned a biological function. Its...
pentameric structure, though, offers the potential for multivalent interactions with other large structural tissue constituents. In this study we show that one such interaction partner for COMP appears to be triple-helical collagen. The strength of the interaction is such that it is likely to be relevant also in the tissue. Furthermore, we demonstrate that it is the globular C-terminal domains of COMP that mediate the interaction, underscoring that a primary functional feature of COMP is multivalent interactions with one or several collagen monomers. It appears that one COMP molecule can concomitantly bind to only one of the binding sites on a particular collagen molecule, as the total length of two subunits of COMP is only 56 ± 6 nm (6). This is too short to span the distance between any two sites on the collagen molecule, although COMP can bridge between such molecules. The novel interaction between COMP and collagens may have several roles in tissue homeostasis, including regulating collagen fibril formation and maintaining the properties and integrity of the collagen network.

REFERENCES
1. Hedbom, E., Antonsson, P., Hjerpe, A., Aeschlimann, D., Paulsson, M., Rosa-Pimentel, E., Sommarin, Y., Wendel, M., Oldberg, A., and Heinegård, D. (1992) J. Biol. Chem. 267, 6132–6136
2. DiCesare, P. E., Hauser, N., Lehman, D., Pasumarti, S., and Paulsson, M. (1994) FEBS Lett. 354, 237–240
3. Smith, R. K., Zunino, L., Webbon, P. M., and Heinegård, D. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 6137–6141
4. Shen, Z., Heinegård, D., and Sommarin, Y. (1995) J. Biol. Chem. 270, 1165–1172
5. Arnold, F. H., and Haymore, B. L. (1991) Science 252, 1796–1797
6. Oldberg, A., Antonsson, P., Lindblom, K., and Heinegård, D. (1992) J. Biol. Chem. 267, 22346–22350
7. Efimov, V. P., Lustig, A., and Engel, J. (1994) Eur. J. Biochem. 223, 937–937
8. DiCesare, P. E., Mörkelin, M., Carlsson, C. S., Pasumarti, S., and Paulsson, M. (1995) Trends Cell. Biol. 15, 513–526
9. Newton, G., Weremowicz, S., Morton, C. C., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., and Lawler, J. (1994) Genomics 24, 435–439
10. Briggs, M. D., Hoffman, S. M., King, L. M., Olsen, A. S., Mohrenweiser, H., Leroy, J. M., Moreau, J., Rine, D. L., Lachen, R. S., Gains, E. S., Cekleniak, J. A., Knowlton, R. G., and Cohn, D. H. (1985) Nature 311, 330–336
11. Benuit, P., and Trencsak, A. (1979) in The Proteins (Neruh, H., and Hill, R. L. eds) pp. 411–632, Academic Press, New York
12. Koch, K. (1987) in Structure and Function of Collagen Types (Mayne, R., and Burgos, R. E. eds) pp. 1–42, Academic Press, New York
13. van der Rest, M., and Mayne, R. (1988) J. Biol. Chem. 263, 1615–1618
14. Vogel, K. G., Paulsson, M., and Heinegård, D. (1984) Biochem. J. 223, 578–597
15. Hedin, L., and Heinegård, D. (1989) J. Biol. Chem. 264, 6898–6905
16. Pringle, G. A., and Dodd, C. M. (1990) J. Histochem. Cytochem. 38, 1455–1441
17. Svensson, L., Heinegård, D., and Oldberg, A. (1995) J. Biol. Chem. 270, 20712–20716
18. Hedin, L., and Heinegård, D. (1995) J. Biol. Chem. 268, 27307–27312
19. Hedin, L., and Heinegård, D. (1995) J. Biol. Chem. 270, 20712–20716