Cartilage oligomeric matrix protein (COMP) and type IX collagen are key structural components of the cartilage extracellular matrix and have important roles in tissue development and homeostasis. Mutations in the genes encoding these glycoproteins result in two related human bone dysplasias, pseudoachondroplasia and multiple epiphyseal dysplasia, which together comprise a “bone dysplasia family.” It has been proposed that these diseases have a similar pathophysiology, which is highlighted by the fact that mutations in either the COMP or the type IX collagen genes produce multiple epiphyseal dysplasia, suggesting that their gene products interact. To investigate the interactions between COMP and type IX collagen, we have used rotary shadowing electron microscopy and real time biomolecular (BLAcore) analysis. Analysis of COMP-type IX collagen complexes demonstrated that COMP interacts with type IX collagen through the noncollagenous domains of type IX collagen and the C-terminal domain of COMP. Furthermore, peptide mapping identified a putative collagen-binding site that is associated with known human mutations. These data provide evidence that disruptions to COMP-type IX collagen interactions define a pathogenetic mechanism in a bone dysplasia family.

The skeletal dysplasias are a diverse group of genetic diseases affecting primarily the development of the osseous skeleton, and range in severity from relatively mild to severe and lethal forms (1). There are over 200 unique well characterized phenotypes (2), and many of these conditions have been grouped into “bone dysplasia families” on the basis of similar clinical and radiographic presentation with the supposition that they will share a common disease pathophysiology (3). While there has been great progress in identifying many of the genes involved in these diseases (4, 5), we still have a very limited understanding of the precise cell matrix pathology of individual phenotypes and the relationship between pathogenetic mechanisms within specific bone dysplasia families.

Pseudoachondroplasia (PSACH)1 and multiple epiphyseal dysplasia (MED) comprise a bone dysplasia family; they are clinically similar diseases characterized by varying degrees of short-limbed dwarfism, joint laxity, and early onset degenerative joint disease (1). Mild and severe forms of PSACH can be recognized (6, 7), and MED presents with considerable clinical variability where traditionally the mild Ribbing and severe Fairbank forms have been used to define the phenotypic spectrum (8).

PSACH results almost exclusively from mutations in the gene encoding cartilage oligomeric matrix protein (COMP) (9–11). COMP is a pentameric glycoprotein found in the extracellular matrix (ECM) of cartilage (12), tendon (13), and ligament, where it is thought to play a major role in tissue development and homeostasis through interactions with cells (14) and other ECM components such as type I and type II collagen (15). It is a member of the thrombospondin gene family (16, 17) and is a modular protein comprising an amino-terminal domain, calcium binding domains (type II and type III repeats), and a large carboxyl domain situated at the distal termini of the pentamer. The majority of the mutations identified in the COMP gene are located within exons encoding the calcium binding type III repeats and are postulated to produce qualitative defects to the protein and a reduction in Ca2+ binding (18). This results in the retention of abnormal COMP pentamers within the rough endoplasmic reticulum (RER) by an undetermined “protein quality control mechanism” (19–22). Interestingly, type IX collagen has been found to colocalize with abnormal COMP in RER vesicles, but the specificity of these intracellular interactions is unknown. Recently, we were the first to identify mutations in one of the exons encoding the carboxyl terminus, thus confirming an important role for this domain in the structure and/or function of COMP (10). Electron microscopy of labrum ligament from a PSACH patient with a COMP mutation shows severe disruption to collagen fibril orientation, variable fibril diameters, and numerous fused fibrils, confirming an important role for COMP in collagen fibrillogenesis.2

Some forms of MED are allelic with PSACH and also result from qualitative defects in COMP (9, 10); however, as a reflection of its clinical variability, MED is genetically heterogeneous

1 The abbreviations used are: PSACH, pseudoachondroplasia; COMP, cartilage oligomeric matrix protein; MED, multiple epiphyseal dysplasia; ECM, extracellular matrix; RER, rough endoplasmic reticulum; HMW, high molecular weight; LMW, low molecular weight; COL, collagenous; NC, noncollagenous; RU, response units; Ct-COMP, C-terminal domain of cartilage oligomeric matrix protein; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; TBS, Tris-buffered saline.

2 P. Holden and M. D. Briggs, manuscript in preparation.
and can result from mutations in the genes encoding type IX collagen (COL9A2 and COL9A3) (23–26). Type IX collagen is closely associated with type II collagen fibrils, where it binds in an anti-parallel orientation to type II collagen molecules (27). Type IX collagen is a member of the FACIT (fibril-associated collagen with interrupted triple helices) group of collagens and is a heterotrimer α1(IX)β2(IX)α3(IX) of polypeptides derived from three distinct genes (COL9A1, COL9A2, and COL9A3). Type IX collagen comprises three collagenous (COL) domains separated by four noncollagenous (NC) domains and has long been thought to act as a molecular bridge between collagen fibrils and other cartilage matrix components (28). The COL3 and NC4 domains project out from the fibrous surface, providing ideal sites for these interactions. All of the mutations identified in the COL9A2 (24, 25) and COL9A3 (23–26) genes are in the splice donor or acceptor sites of exon 3. These result in the skipping of exon 3, leading to an in-frame deletion of 12 amino acid residues from equivalent regions of the COL3 domain of the α2(IX) and α3(IX) chains. The restricted localization of these mutations suggests a possible role for this region of the COL3 domain of type IX collagen in the proposed interactions with other components of the cartilage ECM.

The observations that mutations in COMP (9, 10) and type IX collagen genes (23–26) result in phenotypes within the MCD disease spectrum, that COMP interacts with triple helical type I and type II collagen (15), and that abnormal collagen fibril morphology is associated with COMP gene mutations provide a rationale for investigating potential interactions between COMP and type IX collagen.

In this paper, we show that COMP can interact with type IX collagen. These interactions are mediated through the C-terminal domain of COMP and the noncollagenous domains of type IX collagen. A putative collagen-binding domain in COMP is located between residues 579 and 595, and mutations in this region are likely to disrupt these interactions. Overall, these data provide evidence that disruptions to molecular interactions between two key components of the cartilage extracellular matrix might produce distinct clinical phenotypes that share a common disease pathophysiology and belong to the same bone dysplasia family.

**EXPERIMENTAL PROCEDURES**

**Isolation and Purification of Native COMP from Cartilage—**Fetal bovine calves were obtained fresh from the local slaughterhouse, and articular cartilage was dissected from the large joints. This tissue was then minced in ice-cold TBS, pH 7.4 (20 mM Tris-HCl, pH 7.4, 150 mM NaCl) and briefly homogenized on ice. The homogenate was stirred for 1 h at 4 °C and centrifuged at 10,000 rpm (Beckman JA-10 rotor) for 30 min at 4 °C. The pellet was resuspended in ice-cold TBS, and this process was repeated. To extract COMP, the pellet was resuspended in ice-cold TBS containing 0.1 mM EDTA, briefly homogenized, and stirred overnight at 4 °C. The extract was centrifuged as previously and filtered at 4 °C by gravity flow through Whatman No. 1 filter paper to remove particulate matter prior to chromatography.

The COMP-containing EDTA extract was dialyzed with an equal volume of 20 mM Tris-HCl, pH 7.4, and applied to a 75-ml DEAE-cellulose. The type IX collagen was eluted on a gradient of 20 mM Tris-HCl, pH 7.4, 500 mM NaCl. The column was then washed with 2 column volumes of the same buffer plus 2 column volumes of 150 mM NaCl in the same buffer prior to elution of COMP by application of 2 column volumes of 300 mM NaCl in the same buffer. At the elution stage, fractions of 14 ml were collected, and 20-μl aliquots were analyzed by SDS-PAGE to identify COMP containing fractions. Appropriate fractions were pooled and concentrated by ultrafiltration on ice using an YM-10 membrane (Amicon). The sample was applied to a 24-ml Superose 6 gel filtration column (Amersham Pharmacia Biotech) equilibrated in 20 mM Tris-HCl, pH 7.4, 500 mM NaCl for final purification. Fractions of 0.5 ml were collected, and aliquots of 5 μl from each were analyzed for the presence of COMP by SDS-PAGE and staining with Coomassie blue (Pierce).

**Purification of Recombinant C-terminal COMP—**Total RNA was extracted from human cartilage using TRIZOL® reagent (Life Technologies, Inc.). Approximately 1 μg of total RNA was reverse transcribed using an oligo(dT) primer and superscript reverse transcriptase (Life Technologies, Inc.). An aliquot of cDNA was used for a single step PCR amplification using oligonucleotide primers for the 5′ and 3′ ends of cDNA encompassing the C-terminal domain of COMP (5′-agccgaggctggaggaattc-gaagtcacgctcacc-3′; Ct-R1; 5′-ttcctcgagcggcttgccgcagctgatg-3′). Oligonucleotide Ct-F1 contained an engineered Ascl restriction site, while oligonucleotide Ct-R1 contained an engineered XhoI restriction site. The polymerase chain reaction yielded a product of the expected size (approx. 700 base pairs), which was digested with Ascl and XhoI restriction enzymes and ligated into pSecTag2A vector (Invitrogen) in frame to a polyhistidine tag and Myc epitope. Clones obtained were completely sequenced and a wild-type clone selected for transfection into CHO-K1 cells (ECACC). Cells were grown in Ham’s F-12 medium supplemented with 10% fetal calf serum, and transfaction was performed using Lipofectin® and Opti-MEM® (Life Technologies, Inc.) according to the manufacturer’s protocol. Following transfection, a stable clone was established by selection with Zeocin® at 100 μg/ml (Invitrogen). Resistant cell lines were analyzed for expression of recombinant C-terminal domain of COMP (Ct-COMP) by SDS-PAGE and Western blotting using culture medium using anti-Myc antibody. A clone shown to express recombinant protein was serially diluted to obtain a single cell clone expressing recombinant Ct-COMP, which was then expanded.

**Purification of Recombinant C-terminal COMP—**To simplify purification of Ct-COMP from the culture medium, the concentration of fetal calf serum in the culture medium was gradually reduced from 10 to 2% with no apparent detriment to cell growth/protein production. Cells were grown in 162-cm² TC flasks in medium supplemented with 50 μg/ml Zeocin®. Medium was harvested and replaced at 4-day intervals, and the cells were maintained at near confluence. Harvested medium (100-ml batches) was chilled on ice, and solid (NH₄)₂SO₄ was added slowly with stirring to a final saturation of 50% and stirred at 4 °C for 4 h. The solution was then centrifuged at 10,000 rpm (Beckman JA-10 rotor) at 4 °C, and the supernatant was removed. Further solid (NH₄)₂SO₄ was added to a final saturation of 70% and stirred for 4 h, and the solution was centrifuged as previously. The precipitate was resuspended in Tris-HCl, pH 7.2, 50 mM NaCl, and dialyzed against the same buffer. This was then applied to a 1-ml HiTrap-Q column (Amersham Pharmacia Biotech) equilibrated in the same buffer, and proteins were eluted with a linear NaCl gradient to 500 mM NaCl over 20 column volumes. SDS-PAGE and Western blotting using the anti-Myc antibody identified fractions containing recombinant Ct-COMP. Relevant fractions were pooled and dialyzed against 20 mM Tris-HCl, pH 7.4, 50 mM NaCl, and the solution was applied to a 1-ml Hi-Trap chelating column (Amersham Pharmacia Biotech) charged with Ni₃(SO₄)₂ and equilibrated in the same buffer. Contaminating proteins were washed away, and His-tagged recombinant Ct-COMP was eluted with a linear imidazole gradient to 500 mM over 20 column volumes. 1-ml fractions were collected and analyzed for presence of recombinant Ct-COMP by SDS-PAGE and staining with Gel-code® reagent.

**Purification of Native Type IX Collagens from Cartilage and Vitreous—**Dr. Rod Watson kindly provided type IX collagen isolated from chick sternal cartilage. Briefly, sterna from 50 dozen 17-day chick embryos were incubated in culture medium containing Dulbecco’s modified Eagle’s medium supplemented with 64 μg/ml β-amino propionitrile, 50 μg/ml ascorbic acid, a 1 μg/ml concentration of a uniformly labeled mixture of 14C-telopeptide, and 10 μCi/ml Na₂[³⁵S]SO₄. Type IX collagen was extracted from the sterna in 50 mM Tris-HCl buffer (pH 7) containing 0.2 M NaCl and chromatographed on two consecutive columns of DEAE-cellulose. The Type IX collagen was eluted on a gradient of NaCl, pressure-concentrated, and stored frozen until needed (29, 30). Pepsin-resistant fragments of types II and IX collagen were extracted as pepsin digestion and salt precipitation according to established protocols (31). The pepsin-resistant high molecular weight (HMW) fragments of type IX collagen obtained by this method were separated by molecular sieve chromatography on a Superose-6 column (Amersham Pharmacia Biotech). The short form of type IX collagen was a kind gift from Dr. Kees Jan Bos and was purified from...
bovine adult vitreous by established protocols (32).

SDS-PAGE and Western Blot Analysis—COMP and type IX collagen samples were analyzed in the presence (reduced) or absence (nonreduced) of 100 mM dithiothreitol by SDS-PAGE and stained with Gel-code® (Pierce). Alternatively, proteins were transferred from gels to nitrocellulose by standard protocol, and the nitrocellulose was Western blotted with the relevant primary antibody (1:1000 dilution) followed by the secondary horseradish peroxidase- or alkaline phosphatase-conjugated secondary antibody (1:1000). For each antibody incubation, the diluent was composed of phosphate-buffered saline, 0.1% Tween 20 and 2% (w/v) marvel. Following incubation with the secondary antibody, detection was performed using the enhanced chemiluminescence Western blotting analysis system (Amersham International) or Sigma FAST™ 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium-buffered substrate tablets (Sigma), according to the manufacturer's protocols. Anti-Myc monoclonal antibody was obtained from Roche Molecular Biochemicals, and polyclonal antibodies to type IX collagen were kind gifts from Dr. Paul Bishop. Horseradish peroxidase- and alkaline phosphatase-conjugated secondary antibodies were obtained from Sigma.

Electron Microscopy of COMP-Type IX Collagen Complexes—Either the long form of type IX collagen purified from chich sternal cartilage or the short form lacking the N-terminal NC4 domain purified from bovine vitreous were mixed with native COMP at a molar ratio of 1:1 and dialyzed at 4 °C against 20 mM Tris-HCl buffer (pH 7.4), containing 150 mM NaCl and 1 mM ZnCl2. A modified version of the mica sandwich technique (33) was used to prepare 6-μl aliquots of each sample for platinum carbon rotary shadowing using the Cressington CFE-50B and Nickel 400 TEM grids. Replicas created by this method were studied using a JEOL 1200EX transmission electron microscope operated at an accelerating voltage of 100 kV. Electron micrographs were taken on Agfa Scientia 23Ds6 electron microscope film and then scanned onto a PC using a Polaroid Sprintscan 45 scanner, in preparation for image reproduction or digitized for analysis.

Micrographs obtained were digitized from the photographic film using a monochrome TV camera (Bosch analogue type YK91D) for image analysis purposes. The Semper 6 electron image analysis package (Synoptics Ltd., Cambridge, UK) was used to measure along the length of the collagen molecule to the point of COMP binding, starting at the prominent globular NC4 domain of type IX collagen. Measurements were taken, and the distribution of bound COMP molecules was analyzed by graphing the number of bound molecules relative to defined distances (10-nm intervals) along the collagen molecule.

Surface Plasmon Resonance Assay—Protein-protein interaction studies and peptide competition assays were carried out on the BIAcore™ 1000 or 3000 systems (BIAcore AB, Sweden) as indicated in the figure legends. Native COMP, recombinant Ct-COMP, and BSA were immobilized at 25 °C onto different flow cells of a CM5 sensor chip. The chip surface was first activated by injection of 50 μl of a 1:1 mixture of 0.1 M N-hydroxysuccinimide and 0.4 M N-ethyl-N’-(dimethylaminopropyl)carbodiimide. COMP (100 μl of 15 μg/ml) in 10 mM glycine, 15 mM NaCl, pH 3.0 (optimal buffer components as determined by trial binding experiments) was immobilized at a flow rate of 2 μl/min. Recombinant Ct-COMP (100 μl of 2 μg/ml) was immobilized under the same conditions, and an additional flow cell was prepared as a blank by immobilization of BSA (15 μg/ml) under the same buffer conditions. Remaining activated groups on each flow cell were blocked by injection of 70 μl of 1.0 M ethanolamine HCl, pH 8.5, at a flow rate of 10 μl/min. The system was then primed with 20 mM Tris-HCl, pH 7.4, 150 mM NaCl containing 1 mM ZnCl2. In some binding studies, native type IX collagen at 5 μg/ml, HMW and LMW fragments of type IX collagen at 45 nM, and type II collagen at 200 μg/ml in the same buffer were injected over the flow cell surface at a flow rate of 10 μl/min and a sample volume of 20 μl. In peptide competition assays, collagens were preincubated with peptides at various molar ratios prior to injection under the same conditions. In all studies, upon attainment of a steady sensogram response, the tightly bound proteins were dissociated by injection of 5 mM EDTA in the same buffer (Fig. 1A; otherwise, data not shown). The BIAcore evaluation software version 3.0 was used to compare sensogram readings for the different experiments.

RESULTS

Purification of Native Proteins and Expression of Recombinant C-terminal Domain of COMP—COMP, intact type IX collagen (long and short forms), and the HMW and LMW pepsin-resistant fragments of type IX collagen were extracted from tissue and purified. COMP appeared as a single band when analyzed by SDS-PAGE and Gel-code® staining (data not shown). The HMW and LMW pepsin-resistant fragments of type IX collagen were extracted from bovine cartilage by acid extraction and salt precipitation. Molecular sieve chromatography of the extract resulted in highly pure preparations of these fragments as judged by SDS-PAGE and Gel-code® staining (Fig. 1). The identities of the fragments were also confirmed by Western blot using type IX collagen-specific polyclonal antibodies (data not shown).

To facilitate investigations into the interactions of the Ct-COMP, we developed an expression system for the production of soluble and recombinant Ct-COMP to augment the study of native COMP isolated from fetal bovine tissues. A mammalian expression system using a pSecTag2-Ct-COMP construct was used to produce glycosylated and His-tagged Ct-COMP from CHO-K1 cells. The recombinant protein was isolated from the culture medium by ion exchange and Ni2+ affinity liquid chromatography (Fig. 1). The typical yield of pure recombinant protein from a 100-ml culture was 4 μg.

Interactions between Native COMP and the Long Form of Type IX Collagen Viewed by Rotary Shadowing Electron Mi-
Previously, Rosenberg et al. (15) demonstrated that native COMP binds to type I procollagen, type I collagen, type II procollagen, and type II collagen via its C-terminal globular domain in the presence of Zn$^{2+}$ or Ni$^{2+}$. To determine whether COMP could interact with the long form of type IX collagen under these conditions, we incubated the purified proteins at a 1:1 molar ratio in TBS supplemented with 1 mM ZnCl$_2$. The complexes were prepared for rotary shadowing electron microscopy using a Cressington CFE-50B instrument, and the replicas were viewed by transmission electron microscopy (Fig. 2). The COMP molecules exhibited the characteristic five-armed structure in which the C-terminal domains were clearly visible as globules at the distal ends of the arms. Molecules of type IX collagen appeared as 170-nm-long rods, often exhibiting a pronounced kink about two-thirds along the molecule. In addition, type IX molecules exhibited a distinctive globule, corresponding to the NC4 domain of the molecule.

Images of COMP-type IX collagen complexes were digitized, and the location of the binding site of COMP along each type IX collagen molecule was determined by image analysis. Typically, a type IX collagen molecule was seen to bind one COMP molecule. In some instances, two molecules of COMP could be seen interacting with one type IX collagen molecule. For image analysis, we digitized 172 images of type IX collagen molecules that had one or more bound COMP molecules. To determine the sites of COMP binding, we only used images in which both the COMP and the type IX collagen molecules could be visualized easily (107 in total). In all complexes examined, COMP interacted with type IX collagen via its C-terminal domain, consistent with a single binding site on COMP. However, it was apparent that type IX collagen had more than one binding site for COMP. The results demonstrated that in 80% of the complexes, the C-terminal domain of COMP specifically bound to one of four distinct sites on the type IX collagen molecule (Fig. 3). Careful measurements suggested that these sites corresponded to the noncollagenous (NC1, NC2, NC3, and NC4) domains of type IX collagen (Fig. 3, A–D). The pronounced kink in the type IX collagen molecule at the NC3 domain greatly facilitated assignment of molecular polarity (N to C) of the molecule and precise length and distance measurements. The NC2 domain had the highest frequency of occupation, with 36 from 107 complexes having COMP bound to this domain. The frequencies of binding to the other NC domains were relatively similar to each other (Fig. 3E). We noticed that peaks in the frequency histogram were relatively sharp at the NC1, NC2, and NC4 binding sites (Fig. 3E). However, the peak corresponding to the NC3 domain was relatively broad and ranged from 30 to 70 nm, with a median at 50 nm from the NC4 domain. The broadening of this peak was most probably the result of difficulties in determining the precise binding site because of the abrupt kink in the type IX collagen molecule at the site of binding.

Interactions between Native COMP and the Short Form of Type IX Collagen—The similar dimensions of the NC4 domain of type IX collagen and the C-terminal domain of COMP in rotary-shadowed images made it difficult in some images to assign, unequivocally, binding of COMP to the NC4 domain or to a region in the collagen molecule close to the NC4 domain. Therefore, we repeated the rotary shadowing electron microscopy experiments with the short form of type IX collagen isolated from bovine vitreous, which lacks the NC4 domain (Fig. 4). The NC4 domain is derived exclusively from the α1(IX) chain and through alternative splicing and the subsequent use of an alternative start codon in the COL9A1 gene the vitreous form of type IX collagen lacks the entire α1(IX) NC4 domain (32). The α1(IX) NC4 was clearly missing in all of the type IX collagen molecules observed, and analysis of 25 COMP-type IX collagen complexes failed to identify any COMP molecules binding to the amino terminus of type IX collagen. This confirmed that an interaction with COMP is mediated specifically through the α1(IX) NC4 domain. Bound COMP molecules had a similar distribution between the NC1–3 (Fig. 4E) domains to that seen for the long form of type IX collagen.

COMP-Type IX Collagen Interactions Studied by Real Time Biomolecular Interaction Analysis—In further experiments to examine the association of COMP and type IX collagen, we used real-time biomolecular interaction analysis. The data from these studies provided qualitative evidence that the C-terminal domain of COMP specifically interacted with the noncollagenous domains of type IX collagen.

In the first experiment, native COMP was bound to the surface of a CM5 sensor chip, and when buffer containing type IX collagen was injected onto the chip surface a characteristic sensorgram was recorded (Fig. 5A). There was an initial sharp rise in response units (RU) as a result of refractive index change, and this was followed by a slower association phase.
typical of reversible binding, which reached a plateau on saturation of the binding sites of COMP (labeled A in Fig. 5A). There was then a rapid decrease in RU signal after buffer change (refractive index change), which was followed by the dissociation of type IX collagen that was bound with low affinity (labeled D in Fig. 5A). Finally, an asymptotic phase was observed, which was illustrative of high affinity reversible binding of type IX collagen to the immobilized COMP (labeled B in Fig. 5A). If the BIAcore analysis had been allowed to continue for longer, the sensorgram would eventually have returned to base line once all of the type IX collagen had dissociated. High affinity binding of type IX collagen to COMP was immediately abolished upon the injection of 5 mM EDTA, confirming that the interaction was reversible and cation (Zn$^{2+}$)-dependent (data not shown). This experiment was repeated using several different concentrations of type IX collagen (7.5–15 μg/ml), and the level of binding was seen to be proportional to the analyte concentration (data not shown).

To confirm the specificity of these interactions, we examined the ability of two ECM proteins (fibronectin and laminin) and four other proteins (chosen at random) to bind to COMP. The injection of fibronectin, laminin, BSA, ADH (not shown), β-amylase (not shown), and aproferritin (not shown) over COMP did not exhibit the typical association or dissociation curves (surface plasmon resonance effect) that were observed with type IX collagen (Fig. 5A). In further control experiments, the injection of COMP, type IX collagen, fibronectin, laminin, alcohol dehydrogenase, β-amylase, and aproferritin over BSA bound to the sensor chip failed to show evidence of binding (data not shown).

We considered the possibility that our rotary shadowing EM measurements could have been insensitive to the binding of COMP to the triple-helical regions of type IX collagen immediately adjacent to the noncollagenous domains. Therefore, we performed experiments using BIAcore to qualitatively determine whether there were specific interactions between COMP and pepsin-digested type IX collagen molecules (in which the noncollagenous domains had been proteolytically cleaved). In this series of experiments, we bound COMP to the sensor chip and injected buffer containing purified pepsin-resistant type IX collagen samples, comprising separately the HMW (COL2-COL3) or LMW (COL1) fragments, respectively. In these experiments, the characteristic association and dissociation curves that were seen with intact type IX collagen were seen only, and to a lesser extent, with the HMW fragment and not the LMW fragment (Fig. 5B). In this experiment, the injection of type IX collagen samples was terminated before the attainment of binding saturation, which was done to conserve the limited quantities of type IX collagen we had available. The inability of the LMW fragment to bind to COMP confirms that the NC1 and NC2 domains of type IX collagen contain binding sites for COMP. In the case of the HMW fragment, it has been previously determined that the NC3 domains of human and

**Fig. 3.** COMP binding to the long form of type IX collagen was visualized by rotary shadowing transmission electron microscopy. COMP (36 μg/ml) and the long form type IX collagen (22 μg/ml) were incubated together, at a 1:1 molar ratio, in TBS with 1 mM ZnCl$_2$. The sample was rotary-shadowed using a Cressington CFE-50B freeze fracture instrument and viewed by transmission electron microscopy. Images of individual COMP-type IX collagen complexes showing COMP binding to the NC4 (A), NC3 (B), NC2 (C), and NC1 (D) domains of type IX collagen were collected. A graphical presentation of 107 images of COMP-type IX collagen complexes was created, showing the number of COMP molecules bound at any one point along the type IX collagen molecule (E).
avian \( \alpha_1(IX) \) and \( \alpha_3(IX) \) chains are resistant to digestion by pepsin, while the \( \alpha_2(IX) \) chain is sensitive to pepsin digestion, resulting in a cut between the COL2 and COL3 domains of \( \alpha_2(IX) \) (34, 35). While the extent of cleavage in bovine type IX collagen is unclear, the limited ability of the COL2-NC3-COL3 domain to bind to COMP suggests that the binding site in the NC3 domain is not fully disrupted. Overall, these qualitative data confirmed the rotary shadowing EM observations that binding to COMP was mediated through the noncollagenous domains of type IX collagen.

In further experiments, recombinant Ct-COMP and BSA were immobilized onto different flow cells of the same sensor chip, and native type IX collagen was injected over the surface (Fig. 5C). Typical association (labeled A in Fig. 5C) and dissociation (labeled D in Fig. 5C) phases were seen when type IX collagen was injected over Ct-COMP, indicating that there was reversible binding between type IX collagen and Ct-COMP. The binding of type IX collagen to Ct-COMP was abolished after the addition of 5 mM EDTA (data not shown). Once again the injection of analyte was terminated prior to attainment of binding saturation. These data, in combination with those derived from rotary shadowing electron microscopy experiments, confirmed that COMP binding to type IX collagen was mediated through the C-terminal domain of COMP. The lower response registered on recombinant Ct-COMP (−30 RU) compared with native COMP (−55 RU) resulted from a combination of two factors. First, there was a smaller amount of Ct-COMP bound to the chip surface. This was as a consequence of our expression protocol, which was optimized to produce soluble and glycosylated Ct-COMP rather than maximizing expression. Second, pentameric native COMP consisting of five C-terminal domains per molecule is presumably more likely to present these domains for favorable interactions with type IX collagen at distances away from the chip surface. Conversely, recombinant Ct-COMP molecules would be bound directly to the chip surface in all cases and would therefore be less accessible for interactions with type IX collagen.

Identification of a Potential Collagen Binding Site in COMP—To identify the collagen binding site in COMP, we preincubated type IX collagen with peptides synthesized to regions of the C-terminal domain in which disease-causing mutations had been previously identified. These mutations include E583K (36), T585R and T585M (10), H587R (37), and R718P,3 and the peptides used corresponded to residues 579–595 (peptide 579–595, GVDFEGTFHVNTVTDDD) and 713–723 (peptide 713–723, LDTTMRGGRLG). Preincubation with peptide 579–595 at increasing molar excess (10–1000-fold) re-

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3 S. Unger and D. Cohn, personal communication.
duced proportionally reversible binding of type IX collagen to COMP (Fig. 6A). In contrast, peptide 713–723 at the highest molar excess (10,000-fold) had little effect on binding (Fig. 6B). These data suggest that a putative collagen-binding site, located in the C-terminal domain of COMP, is likely to reside between residues 579 and 595.

Specificity of the Putative Collagen Binding Site—Previously, COMP has been shown to bind to type I and type II...
collagen in the presence of 0.25–2.5 mM Zn\(^{2+}\) (15). To determine whether the binding of COMP to type II collagen and type IX collagen was mediated through the same site in the C-terminal domain of COMP, we preincubated type II collagen with the two peptides prior to passing them over COMP immobilized on the BIAcore sensor chip. Analysis of the sensorogram (Fig. 6C) demonstrated that while preincubation with peptide 713–723 had no effect on the levels of binding of type II collagen to
COMP, preincubation with peptide 579–595 (1000-fold molar excess) reduced binding by a similar extent to that seen with type IX collagen (Fig. 6, compare A and C). These data suggest that the binding of COMP to type II collagen and type IX collagen is mediated through either the same or a closely located binding site.

DISCUSSION

COMP and type IX collagen are important structural components of the cartilage ECM with fundamental roles in collagen fibrillogenesis, tissue development, and homeostasis. We have used rotary shadowing electron microscopy and BIAcore analysis to show that COMP can interact with native type IX collagen. These interactions are mediated through the C-terminal domain of COMP and the noncollagenous domains of type IX collagen (NC1–4). Using BIAcore, we demonstrated qualitatively that COMP can interact with native type IX collagen and to a certain extent the pepsin-derived HMW fragment but not the LMW fragment. These data collectively suggest that each of the noncollagenous domains of type IX collagen are involved in interactions with COMP. The use of recombinant Ct-COMP in BIAcore studies confirmed the rotary shadowing EM findings that the C-terminal domain of COMP mediates interaction with type IX collagen. Furthermore, the use of peptide inhibition assays aided in the identification of a putative collagen-binding site between residues 579 and 595 of COMP. This region of COMP has previously been shown to contain mutations resulting in skeletal dysplasia, providing a direct link between these fundamental interactions and human disease.

Overall, these findings support recent data indicating that the C-terminal domain of COMP can bind to collagen I/II and procollagen I/II molecules in the presence of divalent cations. Using a solid-phase binding assay, Rosenberg and colleagues determined that interactions between COMP and collagen I/II displayed a preference for Zn$^{2+}$, with binding saturated at 0.5 mM. They subsequently characterized these interactions further using BIAcore and rotary shadowing transmission electron microscopy with 1 mM Zn$^{2+}$. We performed similar experiments to study interactions between COMP and type IX collagen, which appear also to be mediated by the C-terminal domain of COMP in the presence of 1 mM Zn$^{2+}$. Whereas Rosenberg and co-workers demonstrated that COMP bound to the collagenous regions of types I and II collagen at four sites located at 0 (C-terminal), 126, 206, and 300 nm (N-terminal), we have shown that COMP appears to bind exclusively to the noncollagenous domains of type IX collagen. We used BIAcore analysis to confirm that type II collagen interacted with COMP in our system and then used peptide inhibition assays to show that this binding could also be specifically disrupted with peptide 579–595 (but not 713–725). These data suggest that type I, II, and IX collagen interactions are mediated through the same (or closely located) region of the C-terminal domain of COMP.

The majority of mutations in the COMP gene are within exons encoding the calcium binding (type III repeat) domain (9–11) and are predicted to result in qualitative defects to COMP (18) leading to the retention of misfolded protein in the RER, a matrix deficient in COMP, and ultimately cell death (21). Interestingly, analysis of chondrocytes from PSACH cartilage shows the accumulation of type IX collagen along with COMP in the RER, suggesting that interactions, possibly specific, occur between these molecules prior to secretion (19). During pentamerization, by random association, 97% of all pentamers will contain at least one abnormal monomer. The relative effect of different numbers of abnormal monomers on COMP pentamer secretion has yet to be determined, but immunohistochemical analysis of cartilage has shown that there is a significant reduction in the level of extracellular COMP that would be available for interactions with collagen (19).

Interestingly, electron microscopy of labrum ligament from a PSACH patient with a mutation in the type III domain of COMP (G465S) shows a generalized disruption to tissue organization and abnormal collagen fibril morphology. Longitudinal sections show severe disruption to the orientation of collagen fibrils and differences in individual fibril thickness, whereas transverse sections show variable fibril diameter, irregular fibril surface, and numerous fused fibrils. Overall, these data confirm a role for COMP in collagen fibrillogenesis and matrix assembly.

We hypothesize that disruptions to COMP-type IX collagen interactions are a secondary component of the pathophysiology of the “PSACH-MED bone dysplasia family.” Disruption to these interactions can occur by one of two mechanisms; either mutations occur within the binding sites of these molecules or there is a reduction in the amount of one (or both) of these molecules in the ECM of cartilage. We have shown that a collagen binding site is located between residues 579 and 595, a region of COMP previously shown to contain mutations that cause either PSACH or MED. Four mutations have been identified within five residues, E583K, T585R, T585M, H587R, (10, 36, 37), suggesting that the motif EGFTH plays an important role in COMP-collagen interactions. We suggest that mutations in exons encoding the C-terminal domain of COMP are likely to have a less deleterious effect on the structure and folding of abnormal COMP, therefore not preventing its secretion into the extracellular matrix. In this case, interactions with type IX collagen are likely to be disrupted by mutations in the collagen-binding site of COMP.

The cell matrix pathology of MED, resulting from either COL9A2 or COL9A3 mutations, is unresolved. Previously, analysis of cartilage ultrastructure has suggested that there was no retention of abnormal type IX collagen within the RER of chondrocytes from some affected patients (38). However, recent data have shown that inclusion bodies can be present with a lamellar structure similar to that seen in chondrocytes from patients with COMP gene mutations (23). Finally, we have reported that a specific mutation in COL9A2 results in the degradation of COL9A2 mRNA from the mutant allele, and undoubtedly this would result in an overall reduction in type IX collagen within the ECM (24). Collectively, these data suggest that although several different mechanisms contribute to the pathophysiology of MED, all of them are likely to result in an ECM deficient in type IX collagen, thus disrupting important interactions between COMP and type IX collagen.

In conclusion, we have shown that COMP interacts with type IX collagen and have identified the domains in each of these proteins that mediate these interactions. High resolution structural studies will be needed to map the binding sites with precision. Disruption to these interactions is likely to define a pathogenetic mechanism in a human bone dysplasia family, and this finding has major implications in understanding the cell matrix pathology of human skeletal dysplasias.

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Cartilage Oligomeric Matrix Protein Interacts with Type IX Collagen, and Disruptions to These Interactions Identify a Pathogenetic Mechanism in a Bone Dysplasia Family
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