Unique Matrix Structure in the Rough Endoplasmic Reticulum Cisternae of Pseudoachondroplasia Chondrocytes

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Mutations in cartilage oligomeric matrix protein (COMP) cause two skeletal dysplasias, pseudoachondroplasia (PSACH) and multiple epiphyseal dysplasia (MED/EDM1). Because COMP exists as a homopentamer, only one mutant COMP subunit may result in an abnormal complex that is accumulated in expanded rough endoplasmic reticulum (rER) cisternae, a hallmark of PSACH. Type IX collagen and matrilin-3 (MATN3), also accumulate in the rER cisternae of PSACH chondrocytes, but it is unknown how mutant COMP interacts with these proteins. The studies herein focus on defining the organization of these intracellularly retained proteins using fluorescence deconvolution microscopy. A unique matrix organization was identified in which type II procollagen formed a central core surrounded by a protein network of mutant COMP, type IX collagen, and MATN3. This pattern of matrix organization was found in multiple cisternae from single chondrocytes and in chondrocytes with different COMP mutations, indicating a common pattern of interaction. This suggests that stalling of mutant COMP and an interaction between mutant COMP and type II procollagen are initiating events in the assembly of matrix in the rER, possibly explaining why the material is not readily cleared from the rER. Altogether, these data suggest that mutant COMP initiates and perhaps catalyzes premature intracellular matrix assembly. (*Am J Pathol* 2007; 170:293–300; DOI: 10.2353/ajpath.2007.060530)

Cartilage oligomeric matrix protein (COMP/TSP5), a large extracellular glycoprotein, is a pentameric member of the thrombospondin gene family.¹ COMP is primarily found in

musculoskeletal tissues and has recently been identified as a marker for osteoarthritis.^{2–10} The function of COMP is unknown, but other thrombospondins demonstrate adhesive properties.¹¹ Recent work suggests that COMP plays an interfacing role by mediating the interactions between cartilage fibrils and the extracellular matrix.^{12,13}

Mutations in COMP cause two skeletal dysplasias, pseudoachondroplasia (PSACH) and multiple epiphyseal dysplasia (EDM1).^{14–17} Many novel mutations have been identified as causing these disorders, with the majority of mutations found in the highly conserved type 3 calcium-binding repeat domain.^{17,18} In comparison, only a few mutations have been identified outside of this region and only in the C-terminal globular domain.¹⁹ The type 3 repeats are highly conserved in all thrombospondins, suggesting that this region is critical for protein function. *In vitro* studies demonstrate that mutations in this region perturb the calcium binding and protein folding resulting in an abnormally long protein subunit.^{20,21} Recent mapping of the COMP mutations in the type 3 repeats and the globular domains on the resolved crystallography model of thrombospondin-1 also suggests that the mutations destabilize the type 3 repeat domain that folds around a calcium core.²²

Long before the gene that causes PSACH was identified, a distinctive cellular phenotype of giant lamellar-appearing rough endoplasmic reticulum (rER) cisternae in PSACH growth plate chondrocytes was described.^{23,24} Identification of mutations in COMP as the cause of PSACH led to the localization of COMP in the giant rER cisternae as well as type IX collagen and matrilin-3 (MATN3).^{23,25–30} Interestingly, mutations in type IX collagen and MATN3 cause four types of multiple epiphyseal dysplasia (EDM2, EDM3, EDM5, EDM6).^{31–37} The fact that mutations in different genes cause a similar clinical phenotype suggests that these proteins play in-

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teracting roles or have similar function(s) in the matrix. Even though the role of these proteins in the extracellular matrix (ECM) seems to be structural, the individual loss of either COMP or type IX collagen or MATN3 does not produce a dwarf phenotype in mice.^{38–41} However, recent work suggests that correct integration and amount of COMP and MATN3 into the matrix depends on the presence of type IX collagen.¹² This finding also suggests that the interaction of these proteins is important for correct matrix assembly, but individual loss of one protein is not sufficient to disrupt normal cartilage development and growth.

In vivo and *in vitro* immunostaining studies have shown that the PSACH matrix is deficient in COMP, type IX collagen, and MATN3 and that these proteins are abundant in the rER cisternae of PSACH chondrocytes.^{26,28} In contrast, type II collagen and aggrecan, the large aggregating proteoglycan, are efficiently secreted with little being retained in the rER.^{26,28} It is unknown why type II collagen is not affected by COMP mutations. In this study, we use fluorescence deconvolution microscopy with image reconstruction and protein modeling to further investigate the effect of different COMP mutations on type II and IX collagens and MATN3, both in the large rER cisternae and the PSACH matrix. We find that type II collagen is the primary structural protein in PSACH pericellular matrix, just as in the wild-type ECM but, importantly, that type II procollagen plays a role in the accumulation of COMP, type IX collagen, and MATN3 into a matrix in the rER cisternae.

Materials and Methods

Chondrocyte and Growth Plate Samples

The samples used in this study were previously described.^{26,28} Normal growth plate (NGP) was used as the control standard to compare the immunostaining of our cultured chondrocyte nodules. Costochondral control and three different PSACH chondrocyte cell lines, with D469del, G427E, and D511Y mutations, were used in these experiments to generate cartilage nodules using our standard laboratory protocol.^{26,28} All cartilage nodules were grown in nonadherent culture conditions for 56 days.

Image Acquisition

Nodules were fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO), embedded in paraffin, and sectioned using standard techniques.²⁶ Tissue sections were deparaffinized and then rinsed in dH₂O for 5 minutes. The sections were serially digested with 1) collagenase (type IA) (Sigma-Aldrich) at 1 mg/ml in phosphate-buffered saline (PBS) for 45 minutes at 37°C; 2) hyaluronidase (type I-S: bovine) (Sigma-Aldrich) at 1 mg/ml in PBS, pH 5.0, for 30 minutes at 37°C; and 3) pepsin A (Sigma-Aldrich) at 1 mg/ml in 0.1 N HCL for 30 minutes at 37°C with 5-minute dH₂O rinses between digestions. Sections were washed in PBS containing 0.05% Tween 20 (PBS-T) for 5 minutes, followed by a 20-minute

incubation with 10% goat serum in PBS-T to reduce non-specific antibody binding. Subsequently, the antibodies were tested individually (data not shown) and then simultaneously incubated on sections at room temperature for 120 minutes: 1) COMP rabbit polyclonal antibody (Kamiya Biomedical, Seattle, WA) at 1:1000, 2) COL2 goat polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:200, and 3) COL9 mouse monoclonal antibody (Iowa Hybridoma Bank, Iowa City, IA) at 1:200. Three fluorescently tagged secondary polyclonal antibodies were chosen from the following: Alexa Fluor 488, 594, 647, and 750 and used at a 1:250 dilution for 30 minutes at room temperature. The sections were washed in PBS-T and incubated with MATN3 antibody (generously provided by Drs. D.A. Hanson and D.R. Eyre, University of Washington, Seattle, WA) at 1:1500 dilution for 120 minutes at room temperature using the Zenon Labeling Alexa Fluor 488, 594, or 750 kits (Molecular Probes, Eugene, OR) following the manufacturer's instructions. Wavelength combinations were selected to visualize all of the proteins in each image acquisition sequence. 4'-6'-Diamidino-2-phenylindole, 100 mg/ml; Molecular Probes) was used to visualize nuclei but was removed in the analysis. Coverslips were mounted using ProLong Gold anti-fade reagent (Molecular Probes) and secured to glass slides. The sections were visualized by scanning from bottom to top of each tissue section on a DeltaVision scanning fluorescence microscope system (Applied Precision, Issaquah, WA) fitted with an Olympus IX70 microscope inverted microscope using a 100-W mercury arc lamp for illumination (Olympus America, Melville, NY) using appropriate excitation/emission filter sets (Chroma Technology Corp., Brattleboro, VT) specific for each of the fluorescent antibodies). Acquisitions were made at a constant gain, set for maximum fluorescent capture. The acquired images (PXL CCD camera; Photometrics, Tucson, AZ) were all taken at $\times 40$ magnification with an aperture of 1.35 (UAPO 340). All images were then deconvoluted for 10 to 15 iterations to remove extraneous fluorescence (SoftWoRx software; Applied Precision), stacked, volume-rendered, and computer modeled with SoftWoRx imaging software as previously described.^{42–44}

Results

Previously, we found that COMP mutations affect the secretion of type IX collagen and MATN3 in PSACH chondrocytes.^{26,28,45} The focus of the current studies was to use a novel imaging approach, fluorescence deconvolution microscopy, to determine 1) whether there is a difference in ECM produced by chondrocytes with PSACH mutations compared with normal chondrocytes, 2) whether there is a specific pattern to the ECM protein retention, and 3) whether it varied by type of COMP. For these studies, we used NGP and control chondrocyte nodules as standards to compare to PSACH chondrocyte nodules with D469del, G427E, and D511Y mutations.^{26,28}

First, we stained sections from NGP, control, and PSACH cartilage nodules to visualize the distribution of

Figure 1. ECM proteins COMP, types II and IX collagen, and MATN3 in the human growth plate and cultured normal and PSACH, G427E, D469del, and D511Y cartilage nodules. Chondrocyte nodules were treated as described previously and in Materials and Methods.²⁶ Proteins are shown: type II collagen (yellow), COMP (green), type IX collagen (red), and MATN3 (blue). Types II and IX collagens, COMP, and MATN3 co-localize in the matrix, indicating similar distributions in the NGP (A–E) and normal cartilage nodules (F–J). Type II collagen is minimally diminished in the PSACH matrices (K, P, U). The three PSACH nodules demonstrate markedly diminished COMP, type IX collagen, and MATN3 staining (L–O, Q–T, V–Y). Intracellular retention of all four proteins can be appreciated and are shown as dots in all of the PSACH chondrocytes. Scale bar = 20 μ m.

four ECM proteins, COMP, types II and IX collagens, and MATN3, using fluorescence deconvolution microscopy. As expected, in the NGP and control cartilage matrices, all of the proteins co-localized to the pericellular matrix and to a lesser extent to the interterritorial and territorial matrices (Figure 1, A–J). For these normal (nonmutant) chondrocytes, no intracellular retention could be appreciated. Only COMP, type IX collagen, and MATN3 are shown in Figure 1, E, J, O, T, and Y, because more than

three channel acquisitions renders the images difficult to interpret. In comparison, the PSACH nodule showed minimal immunostaining of COMP, type IX collagen, and MATN3 in the matrices surrounding the cells (Figure 1, L–O, Q–T, and V–Y, respectively). In contrast, type II collagen was seen in the matrices of all of the nodules (Figure 1, A, F, K, P, and U), although less was appreciated in all of the PSACH matrices (Figure 1, K, P, and U). Intracellular retention of all four proteins, COMP, types II

Figure 2. ECM and expanded rER cisternae in PSACH chondrocytes compared with normal chondrocytes. Chondrocytes and surrounding matrix images were captured and modeled as described in Materials and Methods and presented as wire-frame renditions. Proteins shown: type II collagen (yellow), type IX collagen (red), COMP (green), and MATN3 (blue). One representative chondrocyte is shown in each panel with the nuclei removed from the picture. The NGP (A) and cultured nodule chondrocytes (B) show a layering and interweaving of all four ECM proteins in the matrix only. In contrast, the PSACH chondrocytes (C–E), show abundant type II collagen in the matrix relative to the other three proteins and to normal controls. All of the PSACH chondrocytes have enlarged rER cisternae containing all four proteins, which are depicted by wire frame. Scale bar = 5 μ m.

and IX collagens, and MATN3, was observed in the PSACH chondrocytes with different mutations (Figure 1, K–Y), and the proteins co-localized in cisternae (Figure 1, O, T, and Y) (type II collagen localization not shown). We have previously reported that these proteins also co-localize with calreticulin, a rER resident chaperone protein, demonstrating that these proteins reside in the rER.²⁷ These results are similar to those observed in PSACH patient chondrocyte samples *in vivo* and demonstrate that our nonadherent nodule culture system recapitulates the PSACH chondrocyte cellular phenotype.^{26,28}

To more fully realize the spatial arrangements and interactions of these proteins in the rER cisternae, we captured chondrocyte images from each sample and deconvolved the images. These models are shown as wire-frame renditions to allow visualization of the distribution of the individual proteins in the models (Figure 2).

Again, both the NGP and control nodule chondrocytes showed the expected pericellular and territorial matrix distribution of the ECM proteins (Figure 2, A and B), as discerned previously^{26,28} and in Figure 1. In contrast, the matrix surrounding the PSACH chondrocytes was primarily composed of type II collagen (Figure 2, C–E); COMP, type IX collagen, and MATN3 were markedly diminished in the PSACH matrices compared with the controls. Previously, it has been shown that type II collagen in PSACH chondrocytes is secreted into the ECM and not significantly retained in the rER.^{26,28} Here, we found that type II procollagen was retained in the rER, and this suggested it played a role in ECM protein retention. Most striking was the distinctive and consistent distribution pattern of the ECM proteins in multiple large PSACH rER cisternae within the same chondrocyte (Figure 2D) and chondrocytes with different COMP mutations (Figure 2, C–E). Figure 3 shows detailed renditions of the individual cis-

Figure 3. Arrangement of ECM proteins in the rER of PSACH chondrocytes. Images of rER were captured using fluorescence deconvolution microscopy and modeled as described in Materials and Methods and shown here as wire-frame renditions. Proteins shown: type II collagen (yellow), type IX collagen (red), COMP (green), and MATN3 (blue). rER cisternae from chondrocytes with G427E (A–E), D511Y (F–J), and D469del (K–O) mutations. The retained proteins in the rER have a similar intracellular structure composed of a central core of type II procollagen (A, E, F, J, K, and O) that is surrounded by COMP, type IX collagen, and MATN3 (B–E, G–J, L–O). This pattern is observed with each of the COMP mutations. Scale bar = 2.5 μ m.

Figure 4. Combined wire-frame and solid models of ECM proteins in PSACH rER. The rER cisternae images in Figure 3, J and O, with D469 del and D511Y mutations, respectively, were rotated to visualize COMP, types II and IX collagen, and MATN3. Proteins are shown: type II collagen (yellow), type IX collagen (red), COMP (green), and MATN3 (blue). Cisternae from nodules with D511Y (**A–C**) and D469del (**D–F**) COMP mutations are shown. Wire-frame renditions are in **A** and **D**. Type II collagen is seen as a solid central core in both cisternae (**B**, **C**, **E**, and **F**). Two representative cisternae, demonstrating the matrix network surrounding the type II collagen core, are shown. Two small cisternae are developing, and the network is assembling around the type II procollagen core (**B** and **C**). Scale bar = 2.5 μ m.

ternae from different mutations shown in Figure 2. Close inspection demonstrates a central core composed of type II procollagen (Figure 3, A, E, F, J, K, and O) around which the other matrix proteins co-localized and appear to be in a layered arrangement (Figure 3, B–E, G–J, and L–O). This same pattern was found in all rER cisternae observed regardless of the COMP mutation or the fluorescent wavelength used to visualize each protein (data not shown). Figure 4 depicts the rotated wire model from Figure 3, J (mutation: D511Y) and O (mutation: D469del). Type II procollagen comprised the central core (yellow) whereas the COMP (green), type IX collagen (red), and MATN3 (blue) formed an ordered network surrounding this core (Figure 4, B, C, E, and F). The ordered networks of matrix assembly in each cisterna were identical even though the COMP mutations are different. Smaller type II procollagen cores were present with the COMP, type IX collagen, and MATN3 network being formed (Figure 4, B and C).

Discussion

Mutations in COMP cause a distinctive cellular pathology associated with retention of COMP and other ECM proteins in the rER, which compromises cellular function and ultimately precipitates chondrocyte death.^{28,29} This loss of chondrocytes results in the clinical phenotype of short

stature and structural abnormalities of the bone that contributes to joint abnormalities. Previously, we evaluated COMP, types II and IX collagens, and MATN3 using immunostaining and electron microscopy to describe and localize these proteins in PSACH chondrocytes.^{26,28,29} Here we used fluorescence deconvolution microscopy to evaluate their distribution with respect to each other in the PSACH rER and matrix. These studies were designed to define the organization of COMP, types II and IX collagens, and MATN3 proteins in chondrocytes with different COMP mutations.

Two important observations result from these studies. First, each unique COMP mutation produces a similar organization of protein retention in rER cisternae and second, that an initiating event for this ECM retention might be the development of a type II procollagen core, around which mutant COMP, type IX collagen, and MATN3 are assembled into a layered matrix. All of the individual rER cisternae within the same chondrocyte, and rER cisternae from different patient cells, have this same organization. Thus, regardless of the COMP mutation, each produces the same pattern of intracellular ECM protein assembly.

Previously, we found little type II collagen being retained in the rER cisternae and therefore were surprised to find the type II procollagen core reported here.^{26,28,29} This result most likely is attributable to the greater sensi-

tivity of the fluorescence deconvolution system used here, compared with methods used in previous studies. These studies do not reveal the mechanism by which these proteins interact, but these observations complement other *in vitro* studies suggesting that COMP binds to type II and IX collagens and the matrilins.^{13,46,47} In the PSACH rER, the stalled mutant COMP may bind to, or trap, the type II procollagen.^{48,49} Rosenberg and colleagues¹³ reported that bovine COMP has the capacity to bind type II procollagen *in vitro*. Here we show that mutant COMP associates with procollagen, and both are retained in the rER. However, the process by which the type II procollagen forms the core, or why type II procollagen fibers are not assembled in the matrix network, is not known and cannot be answered in this study. One possibility is that the uncleaved propeptides hinder the assembly of type II collagen into the intracellular matrix because type II collagen is usually assembled into fibers and a fibrillar network after the propeptides are cleaved in the ECM.^{48,49}

Many enlarged rER cisternae were analyzed and each demonstrated this distinctive core with the surrounding meshwork comprised of COMP, type IX collagen, and MATN3. Type II procollagen was not found in the surrounding protein meshwork. This organization is consistent with early reports of lamellar- or punctate-appearing retained protein in PSACH chondrocyte rER cisternae.^{23,50} This intricate network suggests that an ordered matrix is being assembled within the rER PSACH cisternae, and it is likely that this matrix hinders the degradation of mutant COMP. Mutant proteins are usually detected by the cellular quality control mechanism, such as chaperone proteins, that shuttle the mutant proteins for refolding or degradation.^{51–54} Multiple chaperone proteins have been identified as participating in the retention of mutant COMP, and it has been a conundrum as to why mutant COMP is not degraded. These results suggest that COMP may evade degradation because it is integrated into a matrix, and this complex structure cannot be degraded either because the enzymes required to degrade ECM proteins are not present in the rER or the mutant COMP is not accessible to the degradation pathway.

Mutations in COMP; type IX collagen $\alpha 1$, $\alpha 2$, and $\alpha 3$; and MATN3 genes cause six different skeletal dysplasias that are defined by epiphyseal abnormalities, EDM1, EDM6, EDM2, EDM3, and EDM5, respectively, and PSACH (International Nosology and Classification of Genetic Skeletal Disorders–2006 Revision, available at <http://www.isds.ch/Nosology2006.html>).^{17,55} The role of these proteins in the matrix may explain why mutations in any one of them causes perturbation of the growth centers. All of these proteins play a structural role in the ECM, and they have been predicted to interact/bind.^{12,13,46,47,56} The finding of an ordered intracellular matrix in the PSACH rER provides support for this prediction and also suggests that mutations in COMP do not interfere with protein binding. The opposite may be true, however, with mutant COMP actually having a higher affinity for its protein partners, which may potentiate intracellular binding and matrix assembly.

Another important observation is that type II collagen is the primary component of the matrix surrounding the PSACH chondrocytes, which is relatively deficient in COMP, type IX collagen, and MATN3 (Figure 1 and Figure 2, C–E). We have previously shown that aggrecan, the major proteoglycan in the ECM, is not affected by COMP mutations and is relatively unaffected in the ECM compared with COMP, type IX collagen, and MATN3 in the PSACH matrix.^{26,28} However, aggrecan was not evaluated in this study. The observed intracellular interaction of mutant COMP, type IX collagen, and MATN3 leads us to suggest that wild-type COMP forms a similar stable protein network with these protein partners in the ECM. Type II collagen and aggrecan, the two primary components in the PSACH ECM, may not be able to form normal protein interactions because there is a relative deficiency of COMP, type IX collagen, and MATN3. Recent observations in type IX collagen knockout mice suggest that there is poor integration of COMP and MATN3 in the matrix in young null mice, supporting our hypothesis and observations.¹² In addition, EM studies of cartilage nodules with COMP mutations also demonstrate that cartilage fibrils surrounding PSACH patient chondrocytes are disorganized compared with the control matrix, again suggesting that the absence of these proteins affects the integrity of the ECM.²⁹

The unique intracellular matrix may reflect the presence of mutant COMP, which is predicted to comprise 97% of all COMP pentamers synthesized by PSACH chondrocytes and may not represent the true ECM interactions of these proteins. Nevertheless, this finding provides valuable information when considering treatment modalities. For example, as suggested by the mouse model, the presence of COMP is not necessary for normal development, and methods to remove or knock down some of the mutant COMP are being considered. However, this may prove difficult if the knock down is aimed at the proteins, especially if the intracellular matrix structure is assembled rapidly. Techniques aimed at COMP mRNA synthesis may yield better results.

In conclusion, we find that mutant COMP participates in the assembly of a unique matrix structure in large rER cisternae. The architecture of the matrix is the same even when the underlying mutations are different. Mutant COMP is the catalyst for assembling type IX collagen and MATN3 into a matrix that includes a core of type II procollagen. The mutant COMP conformation may drive this premature matrix assembly in the rER rather than the usual location, in the extracellular compartment.

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