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Matrix Biology 20 (2001) 439–450

MATRIX BIOLOGY

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Selective intracellular retention of extracellular matrix proteins and chaperones associated with pseudoachondroplasia

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Received 15 February 2001; received in revised form 25 May 2001; accepted 7 June 2001

Abstract

Mutations in the cartilage oligomeric matrix protein (COMP) gene result in pseudoachondroplasia (PSACH), which is a chondrodysplasia characterized by early-onset osteoarthritis and short stature. COMP is a secreted pentameric glycoprotein that belongs to the thrombospondin family of proteins. We have identified a novel missense mutation which substitutes a glycine for an aspartic acid residue in the thrombospondin (TSP) type 3 calcium-binding domain of COMP in a patient diagnosed with PSACH. Immunohistochemistry and immunoelectron microscopy both show abnormal retention of COMP within characteristically enlarged rER inclusions of PSACH chondrocytes, as well as retention of fibromodulin, decorin and types IX, XI and XII collagen. Aggrecan and types II and VI collagen were not retained intracellularly within the same cells. In addition to selective extracellular matrix components, the chaperones HSP47, protein disulfide isomerase (PDI) and calnexin were localized at elevated levels within the rER vesicles of PSACH chondrocytes, suggesting that they may play a role in the cellular retention of mutant COMP molecules. Whether the aberrant rER inclusions in PSACH chondrocytes are a direct consequence of chaperone-mediated retention of mutant COMP or are otherwise due to selective intracellular protein interactions, which may in turn lead to aggregation within the rER, is unclear. However, our data demonstrate that retention of mutant COMP molecules results in the selective retention of ECM molecules and molecular chaperones, indicating the existence of distinct secretory pathways or ER-sorting mechanisms for matrix molecules, a process mediated by their association with various molecular chaperones.

Keywords: Cartilage oligomeric matrix protein (COMP); Pseudoachondroplasia (PSACH); Extracellular matrix proteins; Chaperones

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0945-053X/01/$ - see front matter
PII: S0945-053X(01)00148-2

1. Introduction

Pseudoachondroplasia (PSACH), an autosomal dominant skeletal dysplasia characterized by short-limbed dwarfism and early onset osteoarthritis, is caused by mutations in the gene encoding COMP (reviewed in Horton and Hecht, 2000). COMP, a pentameric glycoprotein and member of the thrombospondin family of proteins, is found in the extracellular matrix of developing and adult cartilage, and is present in ligament, tendon and synovium (Delot et al., 1998; Di Cesare et al., 1999; Hedbom et al., 1992; Smith et al., 1997). The COMP monomer is composed of several modular domains each of which are thought to fold independently of one other. The amino terminal α-helical coiled-coil domain is important in pentamerization of the COMP molecule (Efimov et al., 1994; Malashkevich et al., 1996), followed by four epidermal growth factor (EGF)-like domains and the thrombospondin (TSP) type 3 domain. The TSP type 3 domain has recently been shown to bind calcium (Chen et al., 2000; Maddox et al., 2000) and is where the majority of the mutations associated with PSACH are found to occur (Ballo et al., 1997; Briggs et al., 1995, 1998; Hecht et al., 1995; Loughlin et al., 1998). Finally, each COMP monomer contains a C-terminal globular domain which has been shown to bind to collagen I and II with high affinity, an interaction dependent on the presence of divalent cations (Adams and Lawler, 1993; Rosenberg et al., 1998). This suggests a role for COMP in the organization of collagen fibrils contributing to tissue structure and function.

The hallmark of PSACH is the presence of enlarged, distinctive lamellar rER vesicles within chondrocytes (Maynard et al., 1972; Stanescu et al., 1982). These have previously been shown to contain COMP and type IX collagen (Delot et al., 1998; Maddox et al., 1997), two components normally expressed and secreted by chondrocytes. Type II collagen was shown to be absent from the ER vesicles in PSACH, indicating a selective process by which specific matrix proteins are either retained or secreted. Large oligomeric glycoproteins such as thrombospondin I and thyroglobulin have been shown to specifically associate with rER proteins during their intracellular processing and secretion (Kuznetsov et al., 1997). These rER proteins most likely function as molecular chaperones in the protein folding process, and they may serve as retention anchors within the quality control system in the secretory pathway for immature or misfolded proteins (Ellgaard et al., 1999; Hammond and Helenius, 1995). Presumably, such factors play a role in the cellular response to a mutant COMP molecule, as in the case of PSACH. Whether the abnormal rER accumulations in PSACH are due to selective interactions with mutant COMP molecules or are the result of a secretory pathway impaired by abnormal COMP is not known. In an effort to better understand not only the pathology of PSACH, but also the selective secretory processes for ECM molecules, we have identified distinct ECM and molecular chaperone proteins retained within the rER inclusions of chondrocytes from a patient with PSACH. These studies suggest a shared secretory pathway whereby intracellular accumulation of mutant COMP molecules results in the selective retention of certain ECM molecules, a process that is mediated by specific molecular chaperones.

2. Materials and methods

2.1. Patient samples

The patient was an 11-year-old female diagnosed with PSACH. She exhibited the typical features of PSACH: short-limb short stature, joint laxity and abnormal radiology. A sample of iliac crest cartilage was removed following informed consent protocol and used as the source of material for subsequent analyses. Tissue samples of iliac crest were also obtained from a 10-year old control for immunofluorescence. The chondrocytes were isolated following collagenase digestion: patient cartilage sample was incubated overnight at 37 °C in Dulbecco's modified Eagle's medium (Life Technologies) containing 5% fetal calf serum and 3 mg/ml collagenase D (Roche). The isolated cells were cultured for four passages.

2.2. Mutation analysis

Total RNA was isolated from the cultured cells with Trizol (Life Technologies) using the protocol provided by the manufacturer. cDNA was generated by reverse transcription using random primers and the Superscript pre-amplification system (Life Technologies). Approximately 1 μg cDNA was subjected to 40 cycles of PCR using Taq polymerase (Promega) with the forward primer 5′-AGTAGCTAGCTG-GTCGCGAC ACTGACCTAGAC-3′ and reverse primer 5′-ATAGTTTAGCGGCCGCTACGTGA CTTCAGCGTTCTCCGG-3′ to amplify the type 3 domain as described previously (Maddox et al., 2000). The 782 bp PCR product was purified using the QIAquick agarose gel extraction kit (Qiagen) and both strands were directly sequenced by automated fluorescent dye terminator method (BigDye, Applied Biosystems, Inc.), using nested primers. The mutation identified on the cDNA was verified on the genomic DNA. Genomic DNA of the proband and her parents was extracted from whole EDTA-collected blood using the QIAmp Blood kit (Qiagen) according to the

manufacturer's instructions. Approximately 500 ng genomic DNA was subjected to 40 cycles of PCR using Taq polymerase with the forward primer HCOMPI12F1 (5′-CGGGTAGCCTTT-GACAAAACG) reverse primer R0-1 (5′-TGGT-TGAGCACCACCCAGTT-3′). The 539 bp products were purified and directly sequenced as described above.

2.3. Antibodies

The rabbit polyclonal antibody to COMP (R3593) was prepared as described previously (Maddox et al., 1997). The rabbit antiserum against HSP47 was a gift from Dr Nagata and is described elsewhere (Hosokawa and Nagata, 2000; Takechi et al., 1994). The mouse monoclonal antibody recognizing aggrecan was purchased from Chemicon (MAb2015). The rabbit polyclonal antibody recognizing decorin (LF 113) was a gift from Larry Fisher (Fisher et al., 1995). The fibromodulin antibody was a gift from Dr A. Plaas described in (Plaas and Wong-Palms, 1993). The mouse monoclonal antibody that recognizes type II collagen (CIICI) was obtained from the Developmental Studies Hybridoma Bank. Antibody to type VI collagen (R279) was a rabbit polyclonal, used as described in (Keene et al., 1998). The type IX and type XI collagen antibodies are rabbit polyclonals (R9264, and R16123) prepared and used as described previously (Davies et al., 1998; Keene et al., 1995; Morris et al., 2000). Rabbit polyclonal antiserum raised against type XII collagen was used as described previously (Keene et al., 1991). The antibodies recognizing cyclophilin B (PA1-027) and PDI (MA3-019) were purchased from Affinity Bioreagents, Inc. (ABR), the antibody recognizing calnexin was purchased from Stressgen (SPA-860) and the antibody recognizing grp78 was a goat polyclonal antibody (SC-1050) purchased from Santa Cruz Biotechnology. The antibody recognizing ubiquitin was a mouse monoclonal (MAB1510) and was purchased from Chemicon.

2.4. Immunofluorescence

Frozen sections of 8 μm in thickness were made on tissue samples from the patient and from normal age-matched control cartilage. Sections were fixed with 4% paraformaldehyde in PBS for 1 h at 25 °C and then digested with chondroitinase ABC (Sigma) at a concentration of 0.2 units/ml in PBS at 25 °C for 3 h. Sections were washed in 100 mM Tris, pH 7.6 and 0.1% Triton, followed by 100 mM Tris, pH 7.6, then blocked for 30 min at 25 °C in 2% goat serum and incubated overnight at 4 °C with primary antibodies. Sections were washed in 100 mM Tris, pH 7.6 and incubated in goat anti-mouse or goat anti-rabbit IgG antibodies conjugated with FITC (Sigma) at 25°C for 2 h. They were stained with propidium iodide (Molecular Probes) to localize nuclei and then coverslipped in SlowFade Antifade medium (Molecular Probes). Immunofluorescent images were obtained either with a Zeiss Axiophot fluorescence microscope as color slides, which were then digitized, or with a Nikon E800 microscope equipped with a Sensys digital camera (Photometrics, Tucson, AZ) and utilizing Metamorph software (Universal Imaging, Wester, PA).

2.5. Electron microscopy

Tissue samples included iliac crest from the 10-year old proband, navicular growth plate from a 5-year-old control and distal femur from a 15-year-old control. Some cartilage was prepared for electron microscopy by fixation in 1.5% paraformaldehyde and 1.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 containing 0.05% tannic acid and 0.1% $CaCl_2$. The samples were rinsed in buffer then immersed in 1.0% buffered OsO_4, dehydrated in a graded series of ethanol to 100%, infiltrated in propylene oxide and embedded in Spurr's epoxy. Other tissue was fixed in cold 0.1% glutaraldehyde/4% paraformaldehyde for 30 min, dehydrated at progressively lower temperatures (0–20°C), infiltrated in LR White media, and polymerized at 60°C. Sections were mounted on formvar coated nickel grids and floated sequentially on 0.15 M Tris–HCl, pH 7.5, blocking agent (0.05 M glycine in Tris, 2% NFDM containing 0.5% ovalbumin and 0.5% fish gelatin in Tris) and incubated in primary antibody (diluted 1:10 in Tris) for 2 h. Sections were rinsed several times in Tris, incubated with a combination of 5 and 10-nm gold conjugated secondary antibody (1:10 in Tris with 0.5% ovalbumin, 90 min) and given a final rinse in Tris. Labeled sections were examined either stained in uranyl acetate and lead citrate or unstained (Sakai and Keene, 1994).

3. Results

3.1. Mutation analysis

Tissue from the PSACH patient was extracted and analyzed for mutations in the COMP gene. Direct sequencing of a PCR fragment from exon 14 in the patient showed a heterozygous A/G peak at position 1544 of the coding sequence (GenBank accession no. L32137). This base substitution is predicted to result in a change from an aspartic acid to a glycine residue at position 515 in the amino acid sequence (Fig. 1). Blood samples from the unaffected parents were analyzed and the DNA was found to carry only the normal COMP sequence indicating that PSACH in

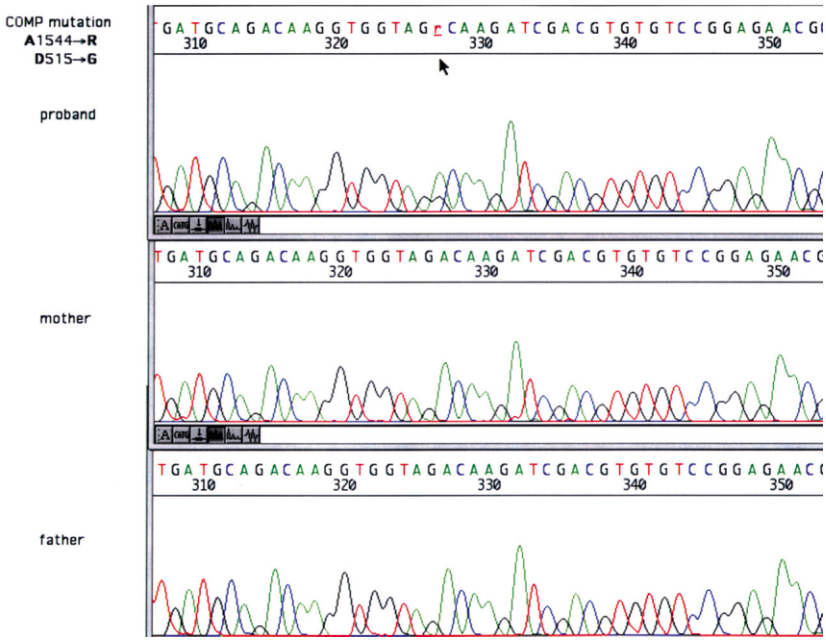

Fig. 1. Mutational analysis of DNA extracted from PSACH cartilage (proband) and unaffected parents. Direct sequencing of PCR fragment of the *COMP* gene showed a heterozygous A/G peak at position 1544 in the coding sequence (arrow). Both parents had a single A peak at the same position.

this patient was caused by a spontaneous mutation in the COMP gene. The D515G point mutation is located near the carboxy terminal end of the TSP type 3 domain of COMP, a domain consisting of a contiguous set of calcium binding sites rich in aspartic acid residues.

3.2. Ultrastructural characterization

Patient and control cartilage was prepared to reveal optimal ultrastructure and examined in the electron microscope. Patient chondrocytes typically contained abnormally distended rER, although the appearance of these inclusions varied considerably and was heterogenous in terms of typical lamellar patterning. Frequently, rER inclusions were seen to occupy a majority of the intracellular space within individual chondrocytes, often with more than one large rER inclusion per cell, and thus affected the overall cell morphology (Fig. 2a). Recurrently, the inclusions within chondrocytes residing in the resting and proliferative zones were non-lamellar and contained dense spherical bodies (Fig. 2a). Inclusions within other chondrocytes in the same region appeared intermediate in lamellar morphology (Fig. 2b). Within hypertrophic chondrocytes of the patient, most inclusions were large and distinctly lamellar. No similar inclusions were observed in chondrocytes of control cartilage, although the rER was occasionally distended. The apparent metabolic condition of the non-hypertrophic chondrocytes also varied considerably. Often PSACH chondrocytes contained regions of normal rER and golgi adjacent to the large lamellar bodies within the same cell (Fig. 3a). Regions of narrow yet metabolically active rER appeared to connect to the larger rER inclusions (Fig. 3b) suggesting the possibility that they are the intermediates in a stepwise progression to formation of the larger intracellular structures.

3.3. Intracellular retention and preferential localization to rER inclusions

In an effort to determine whether other molecules are retained abnormally in the rER inclusions in PSACH, we analyzed by immunofluorescence and immunoelectron microscopy the pattern of antibody localization first to ECM proteins in cartilage from the patient with PSACH and from normal, age-matched controls. In control cartilage COMP (Fig. 4A) was localized to the interterritorial matrix surrounding the chondrocytes by immunofluorescence. In PSACH, COMP was found to be retained intracellularly both by immunofluorescence (Fig. 4B) and by immunogold labeling (Fig. 4C), and was shown to specifically localize within the enlarged rER inclusions by the latter method, as has been reported previously (Delot et al., 1998; Maddox et al., 1997). COMP staining was also present at low but detectable levels in the extracellular matrix in PSACH cartilage, a finding different

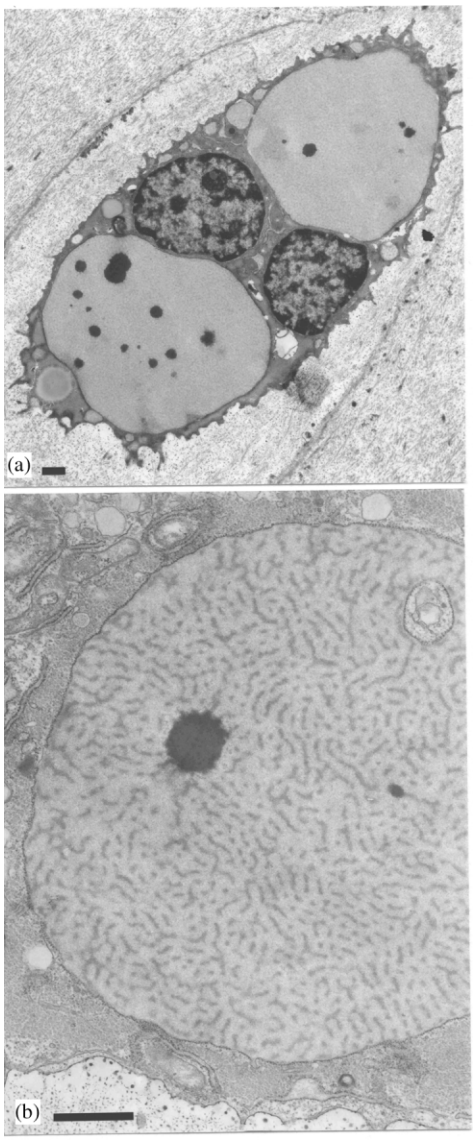

Fig. 2. Electron micrograph of a chondrocyte from PSACH patient cartilage. Enlarged rER inclusions occupy a large volume of the cell and affects overall cell morphology (a). Higher magnification of a PSACH chondrocyte (b) shows enlarged rER inclusion with intermediate lamellar appearance and dense, spherical bodies of unknown composition. (Scale bars = 1 μm.)

from what has been published previously (Maddox et al., 1997) and which may be due to the difference in mutation. Additionally, we examined the antibody staining and localization for an ER-specific protein. In normal chondrocytes, antibody staining for HSP47, a collagen-specific ER-resident molecular chaperone (Hosokawa and Nagata, 2000; Nagata, 1996), was moderate, and showed intracellular localization typical for an ER protein (Fig. 4D). In PSACH chondrocytes staining for HSP47 was increased by immunofluorescence (Fig. 4E) and was found to localize preferentially to the enlarged rER inclusions by immunoelectron microscopy (Fig. 4F). It also appeared that the overall cell size was increased in the PSACH chondrocytes containing the rER inclusions (Fig. 4E) as compared with the cells in normal tissue (Fig. 4D).

3.4. Selective intracellular retention of matrix molecules

To further define the enlarged rER inclusions in PSACH we examined antibody staining and localization of the collagen molecules normally found in cartilage (reviewed in Buckwalter and Mankin, 1998).

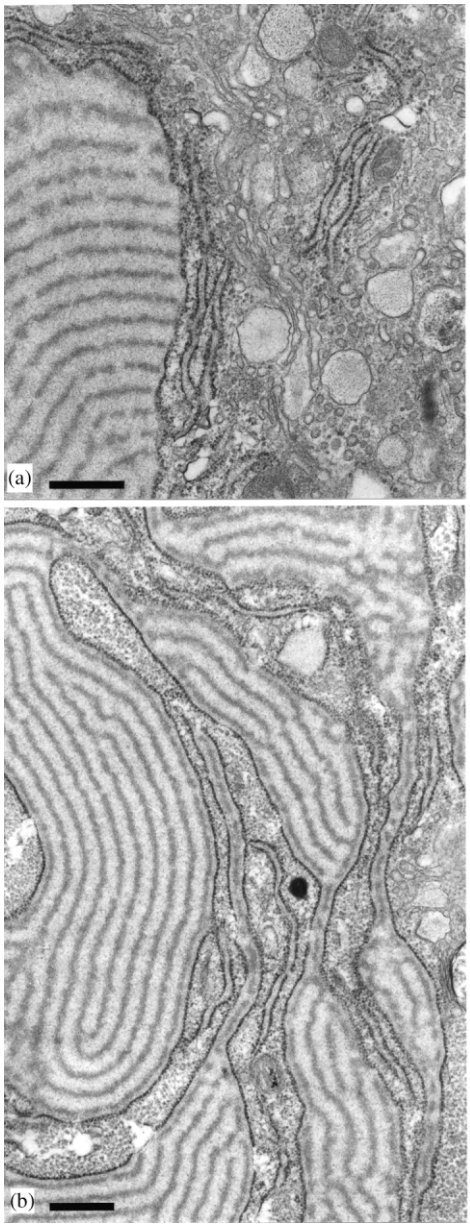

Fig. 3. Electron micrographs of rER inclusions in PSACH chondrocytes at higher magnification. Normal rER and golgi are within the same cell and adjacent to the abnormally enlarged rER inclusions (a). Lamellar appearance of rER inclusions is present throughout ER structures of varying thickness and some inclusions are interconnected (b). (Scale bars = 500 nm.)

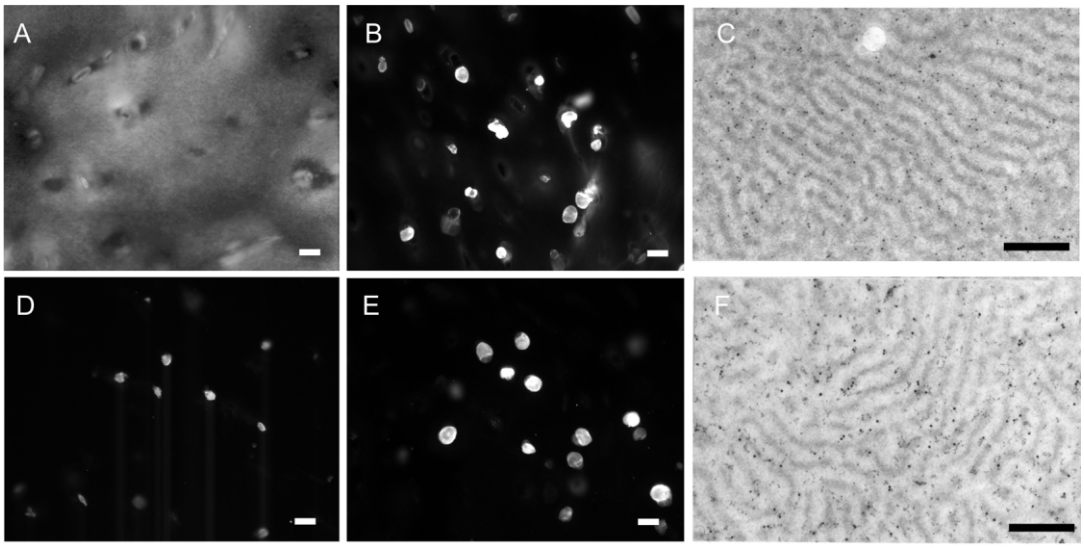

Fig. 4. Immunofluorescence (A, B, D, E) and immunoelectron microscopy (C, F) of cartilage from normal, age-matched control cartilage (A, D) and from the PSACH patient (B, E). Antibodies recognizing either COMP (A–C), HSP47 (D–F) were used for localization in tissues. COMP in control cartilage is extracellular (A) but intracellular in PSACH cartilage (B) and specifically localizes to the rER inclusions (C). HSP47 is intracellular in control and PSACH cartilage (D, E) and specifically localizes to the rER inclusions in PSACH chondrocytes (F). (Scale bars = 20 μm in panels A, B, D and E. Scale bars = 500 nm in panels C and F.)

In comparison with strong, intracellular staining for COMP within the rER vesicles of the patient tissue (Fig. 5A), type II collagen stained only the extracellular matrix of PSACH cartilage (Fig. 5B). Immunofluorescent staining for type VI collagen in patient tissue (Fig. 5C) was similar to what has been shown previously in normal cartilage, namely that it is extracellular and immediately adjacent to chondrocytes forming part of the pericellular matrix (Keene et al., 1998). In agreement with these results, immunoelectron microscopy using the same antibody to type VI collagen only showed extracellular labeling in PSACH tissue (data not shown). In contrast, type IX collagen, a fibril-associated and cartilage-specific molecule that is normally found at low levels in the territorial region surrounding chondrocytes, was strongly localized within the patient cells by immunofluorescence (Fig. 5D). Type IX collagen was shown to be preferentially located within the rER inclusions by immunoelectron microscopy, as has been demonstrated previously (Delot et al., 1998; Maddox et al., 1997). Type XI collagen, a fibrillar collagen and minor component of mature cartilage that normally associates with type II collagen, was localized at low levels within the rER inclusions of PSACH chondrocytes (Fig. 5E). Some extracellular staining was also detectable by immunofluorescence. Type XII collagen is another fibril-associated collagen detected at very low levels surrounding chondrocytes in normal cartilage. Staining for type XII collagen in PSACH cartilage was similar to that for type XI collagen, present at low but detectable levels within the rER of chondrocytes (Fig. 5F). Immunogold labeling using the same antibodies was in agreement with the immunofluorescent data showing low levels of labeling for types XI and XII collagen and high levels of type IX collagen within the rER inclusions of PSACH chondrocytes, whereas type VI collagen was secreted out into the cartilage matrix.

Since specific proteoglycans have been shown to interact with collagens in cartilage (Hagg et al., 1998; Hedbom and Heinegard, 1993), further investigations were carried out to determine whether these proteoglycans were also present in the rER inclusions of PSACH chondrocytes. Aggrecan, the predominant proteoglycan in cartilage, was located throughout the extracellular matrix and in the matrix adjacent to chondrocytes in control cartilage as well as PSACH cartilage, by immunofluorescence (Fig. 5G). In contrast to previously published results (Stanescu et al., 1993), the rER inclusions in the PSACH tissue did not appear to contain aggrecan, especially in comparison to the high levels of COMP staining (Fig. 5A) within the same cells. Further analysis of aggrecan labeling by immunoelectron microscopy with the same antibody confirms these results by demonstrating its presence only in the ECM of PSACH cartilage (Fig. 6). Moreover, aggrecan is not present above background labeling within the enlarged rER vesicles of PSACH cartilage. The small proteoglycans decorin and fibromodulin, integral components of articular cartilage that interact with fibrillar collagens, were localized at moderately high levels throughout the extracellular matrix in control cartilage. In PSACH cartilage, both decorin (Fig. 5H) and fibromodulin (Fig. 5I) were not only present in the extracellular matrix, but also detected intracellularly in the rER

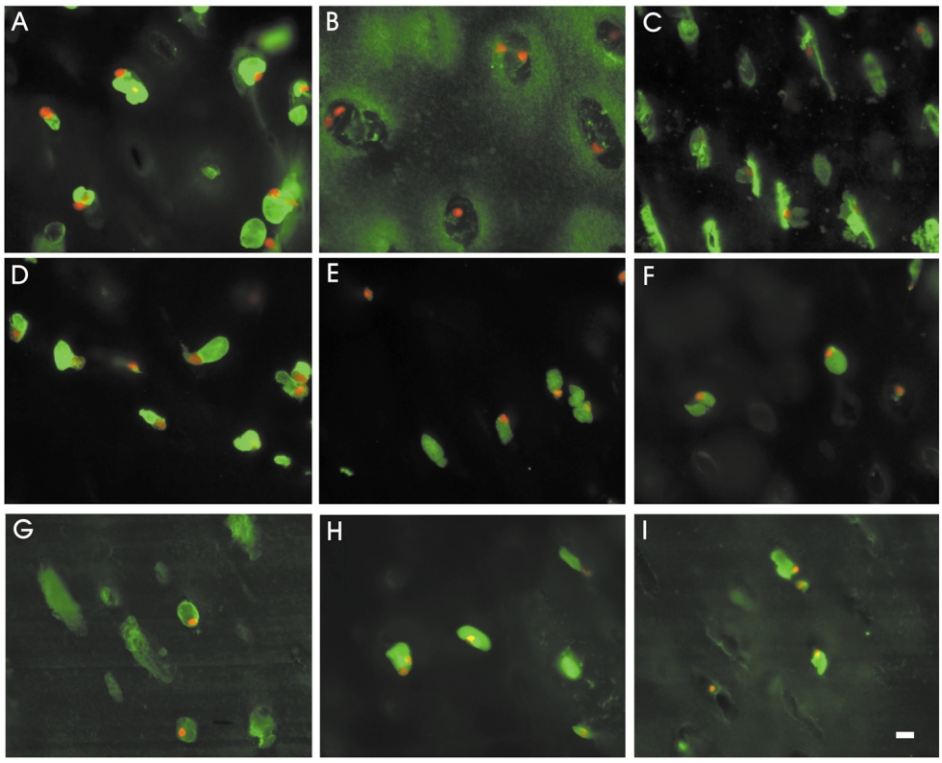

Fig. 5. Immunofluorescence of PSACH cartilage with FITC-labeled secondary antibodies (green stain) and propidium iodide in the nucleus (red stain). COMP (A) is localized within the chondrocytes and to the rER inclusions. In contrast, type II collagen (B) is localized to the cartilage matrix. Type VI collagen (C) is localized to the pericellular regions of the cartilage matrix and is not retained within the cells. Type IX collagen (D) is very strongly localized to the rER inclusions of PSACH chondrocytes. Type XI (E) and type XII (F) collagen are both localized within the PSACH chondrocytes at low levels. Immunostaining for aggrecan (G) was detected only in the cartilage matrix of PSACH tissue, whereas decorin (H) and fibromodulin (I) were detected at moderate levels within the rER inclusions of PSACH chondrocytes and at low levels in the cartilage matrix. (Scale bar = 10 μm and is the same for all panels in figure.)

inclusions at moderate levels by immunofluorescence. Immunoelectron microscopy using the same antibodies to decorin and fibromodulin was consistent with these findings and showed specific localization of the small proteoglycans to the rER inclusions (data not shown). These results demonstrate that retention of mutant COMP molecules is associated with the selective retention of other matrix molecules, including type IX collagen, fibromodulin, decorin and low level amounts of types XI and XII collagen, whereas aggrecan and types II and VI collagen appear to be secreted normally.

3.5. Intracellular concentration of molecular chaperones

To further characterize the apparent secretory defect of mutant COMP molecules in PSACH, we investigated whether typical ER resident proteins that are normally involved in the quality control mechanism of the secretory pathway were also present in the abnormally enlarged rER inclusions. Antibodies recognizing various ER-chaperones were used for immunofluorescence and immunoelectron microscopy. Included were antibodies to HSP47, the collagen-specific molecular chaperone (Nagata, 1996), protein disulfide isomerase (PDI), an ER-resident protein with isomerase and chaperone activities (Wang, 1998) and calnexin, an integral membrane protein of the ER that binds glycoproteins and functions as a chaperone (Bergeron et al., 1994; Tatu and Helenius, 1997). Also included were antibodies to grp78 (BIP), a major chaperone of the ER lumen, and cyclophilin B, an ER-localized peptidyl prolyl *cis-trans*-isomerase (PPIase). All of the chaperone proteins analyzed were

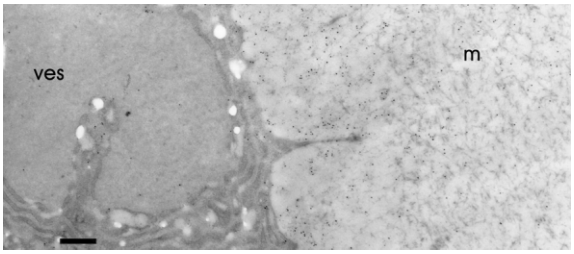

Fig. 6. Immunoelectron microscopy of PSACH chondrocyte with the antibody recognizing aggrecan. Aggrecan is localized at high levels in the extracellular matrix (m) surrounding the chondrocytes but is not present above background levels within the enlarged rER vesicles (ves) of the PSACH chondrocytes. (Scale bar = 500 nm.)

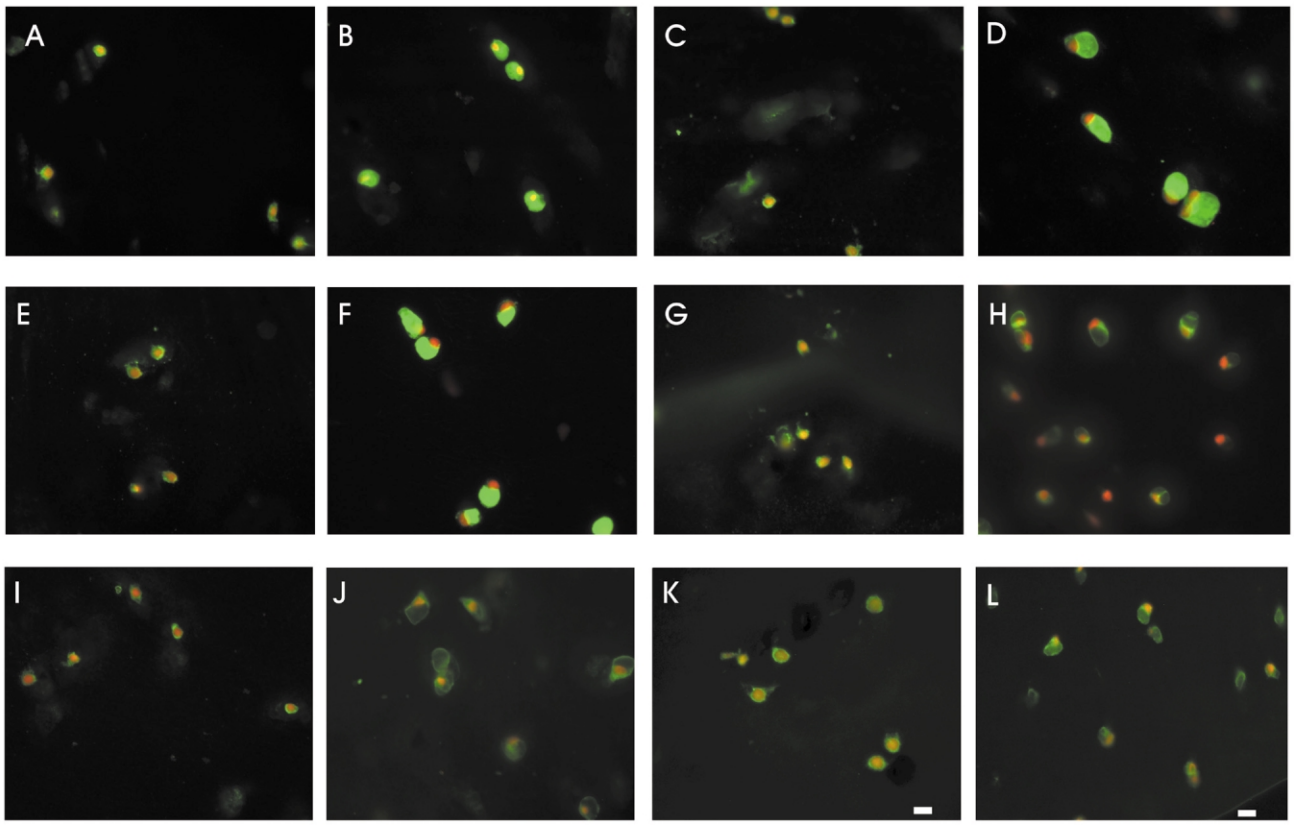

Fig. 7. Immunofluorescence on age-matched control cartilage (A, C, E, G, I and K) and PSACH cartilage (B, D, G, H, J and L) with FITC-labeled secondary antibodies (green stain) and propidium iodide in the nucleus (red stain). Staining for calnexin (A, B) is strongly detected and appears to stain much of the cell including the rER inclusions of PSACH chondrocytes (B). Immunostaining for HSP47 (C, D) is localized adjacent to the nucleus in the control cartilage (C), and is strongly detected within the enlarged rER inclusions of PSACH chondrocytes (D). Immunostaining for PDI is similar to that of HSP47 in that it is detected in the cytoplasm of control chondrocytes (E) and at increased levels within the enlarged rER inclusions of PSACH chondrocytes (F). Immunostaining for both cyclophilin B (G, H) and grp78 (BIP) (I, J) is detected intracellularly in control (G, I) as well as in PSACH chondrocytes (H, J). However the staining levels for both cyclophilin B and grp78 are relatively low in PSACH chondrocytes and both proteins appear to be absent from the enlarged rER inclusions, as determined by relative fluorescence intensity. Intracellular immunostaining for ubiquitin (K, L) is detectable in control chondrocytes (K) as well as in PSACH chondrocytes (L), but also appears to be not present at significant levels within the rER inclusions of PSACH chondrocytes. (Scale bars = 10 μm and is the same for all panels in figure.)

localized intracellularly to the ER at moderate levels in control cartilage by immunofluorescence, as is illustrated in Fig. 7, with antibodies to calnexin (Fig. 7A), HSP47 (Fig. 7C), PDI (Fig. 7E), cyclophilin B (Fig. 7H), grp78 (Fig. 7I) and ubiquitin (Fig. 7K). In PSACH, chondrocytes staining for calnexin (Fig. 7B), HSP47 (Fig. 7D) and PDI (Fig. 7F) was significantly increased and present specifically within the enlarged rER inclusions by immunofluorescence. The levels of staining of these three chaperone proteins in the rER inclusions from PSACH cartilage, as determined by immunofluorescence, was comparable in intensity to that of COMP staining. In contrast, immunostaining for cyclophilin B (Fig. 7H) and grp78 (Fig. 7J) was detectable but appeared to be absent from the enlarged rER inclusions in PSACH chondrocytes. Immunoelectron microscopy with the antibodies recognizing calnexin and HSP47 further support the immunofluorescence data showing the presence of calnexin (Fig. 8a) and HSP47 (Fig. 8b) within the enlarged rER vesicles of PSACH chondrocytes. Calnexin appears to be present in the cytoplasm of the PSACH chondrocytes as well as in the enlarged rER vesicles (Fig. 8a), whereas HSP47 appears to be localized predominantly to the enlarged rER vesicles (Fig. 8b).

The fact that mutant COMP and other matrix molecules are abnormally retained within rER inclusions indicates that they are not targeted for proteosomal degradation as has been shown to occur with other abnormally folded proteins (reviewed in Bonifacino and Weissman, 1998). Ubiquitination is one mechanism by which misfolded proteins are targeted for proteolytic degradation via the 26S proteosome, a process requiring ER-retained proteins to be retrotranslocated to the cytosol (reviewed in Ciechanover et al., 2000; Sommer and Wolf, 1997; Wilkinson, 1999). To determine whether ubiquitin was associated with

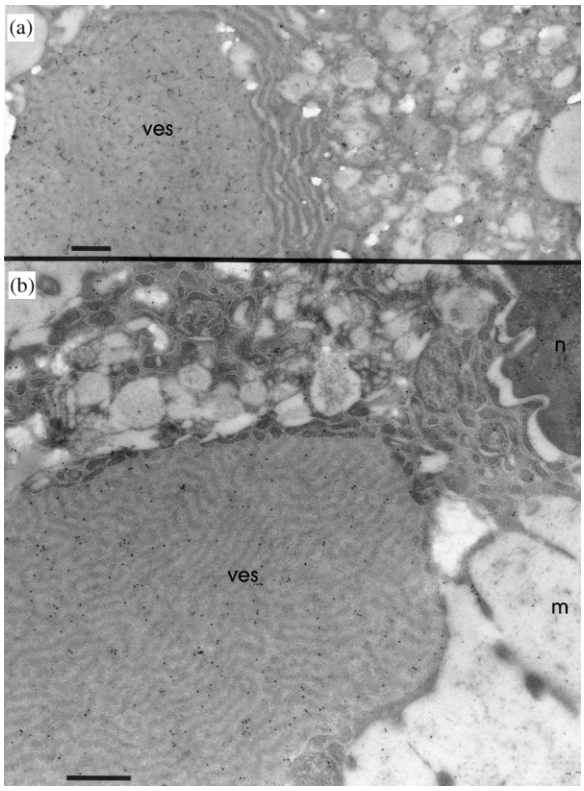

Fig. 8. High resolution immunoelectron microscopy with antibodies to calnexin (a) or HSP47 (b) on PSACH chondrocytes. Immunogold labeling demonstrates the presence of calnexin within the enlarged rER vesicle (ves) as well as in the cytoplasm of the patient chondrocytes (a). Immunogold labeling of HSP47 shows that it is also present within the enlarged rER vesicles of PSACH chondrocytes but does not appear to localize to the cytoplasm, nucleus (n) or extracellular matrix (m) of the cells. (Scale bars = 500 nm.)

abnormally retained matrix proteins within the rER inclusions in PSACH cartilage, we examined its intracellular localization by immunofluorescence. In control cartilage, ubiquitin was expressed at moderately low levels in the cytoplasm of chondrocytes (Fig. 7K). In PSACH chondrocytes, ubiquitin appeared to be present at similar levels within the cells, but was absent from the rER inclusions (Fig. 7I). This suggested that the mutant and presumably misfolded COMP molecules were escaping ubiquitination and thus the normal progression to ER-associated degradation by the proteosome.

4. Discussion

In this study we report a novel D515G mutation in the calcium-binding TSP type 3 domain of COMP in a patient diagnosed with PSACH. It is in this region of the protein where the majority of reported PSACH mutations have been found to occur, demonstrating the importance of calcium binding to the structure and function of COMP. Although the function of COMP remains unclear, the mutations associated with PSACH presumably interfere with the protein's ability to bind calcium thereby affecting the overall structure and function of the protein. Distinct and enlarged rER accumulations within PSACH chondrocytes are a characteristic of the disease, and have previously been shown to contain COMP and type IX collagen (Delot et al., 1998; Maddox et al., 1997). In these studies, we demonstrate selective retention of not only COMP and type IX collagen, but also other matrix molecules within the rER inclusions in PSACH chondrocytes suggesting that multiple secretory pathways may exist for extracellular matrix proteins. Additionally, distinct molecular chaperones were found at elevated levels within the abnormal rER inclusions in PSACH chondrocytes indicating their involvement in the retention of mutant COMP molecules.

Cartilage from the patient was analyzed by electron microscopy and shown to contain chondrocytes with a heterogeneous population of rER inclusions. These intracellular structures varied in their size and appearance and not all contained the typical lamellar pattern of staining. Frequently, the PSACH chondrocyte inclusions within the resting and proliferative zones were non-lamellar, whereas most inclusions in the hypertrophic chondrocytes were large and lamellar. Importantly, PSACH chondrocytes containing the enlarged rER vesicles were generally much larger in overall size with the rER vesicles often occupying a large proportion of the cell volume and thus altering the cell morphology. When antibodies to COMP and HSP47, the collagen-specific chaperone were used for immunolocalization in the PSACH chondrocytes, the rER inclusions were found to contain elevated levels of both proteins as compared with age-matched control cartilage.

Further analysis of PSACH cartilage was performed by immunolocalization of various matrix proteins in order to determine whether they were retained along with mutant COMP or secreted normally. Type II and VI collagen were found to be secreted normally from the patient chondrocytes as determined by immunofluorescence. Data shown here are in agreement with previous results demonstrating that type IX collagen is retained at high levels within the rER inclusions (Delot et al., 1998; Maddox et al., 1997). Type XI and XII collagen were found to reside within the rER inclusions at much lower but detectable levels by both immunofluorescence and immunoelectron microscopy. In addition, various proteoglycans known to reside in the cartilage matrix were analyzed in PSACH tissue. In contrast to previously published results (Stanescu et al., 1993), we found that aggrecan was not retained within the rER inclusions in PSACH chondrocytes but was secreted

out into the cartilage matrix. This was demonstrated clearly by both immunofluorescence and immunoelectron microscopy. In contrast, the small proteoglycans fibromodulin and decorin were found to be present within the rER inclusions at significant levels. These observations demonstrate that in PSACH cartilage there is selective intracellular retention of mutant COMP, types IX, XI and XII collagen, decorin and fibromodulin. We hypothesize that different secretory pathways or different ER sorting mechanisms exist for various matrix molecules, which may help to explain why some matrix molecules are not abnormally retained within PSACH chondrocytes. This hypothesis is supported by earlier work from Vertel et al. (1989), who showed segregation of type II collagen and aggrecan into distinct ER sub-compartments during their progression through the secretory pathway. The pathology of PSACH chondrocytes may thus be due to compartmentalization of mutant COMP and other matrix molecules during their secretion. Increased local concentrations of mutant COMP, as a result of ER retention, may facilitate interactions with other matrix molecules, especially with those usually found to occur in the cartilage matrix, resulting in the formation of organized protein aggregates within the rER inclusions.

Mechanisms of quality control within the ER are crucial for the proper folding, assembly and processing of proteins destined for secretion. Misfolded and incompletely assembled proteins are known to be retained within the ER and are subsequently targeted for degradation, a process referred to as ER-associated degradation (Bonifacino and Weissman, 1998; Ellgaard et al., 1999). This process has been shown to involve interactions with a variety of molecular chaperones and folding enzymes that normally reside in the ER. In order to determine whether specific ER proteins were involved in the selective retention of distinct matrix proteins and mutant COMP molecules, we stained control and PSACH cartilage with antibodies to calnexin, HSP47, PDI, cyclophilin B and grp78 (BIP). Our results show that HSP47, PDI and calnexin were all detected at significantly elevated levels in the rER accumulations of PSACH chondrocytes as indicated by relative intensities of immunofluorescence staining. Immunoelectron microscopy with antibodies to calnexin and HSP47 confirmed their presence specifically within the rER inclusions of PSACH chondrocytes. In contrast, cyclophilin B, an ER-resident chaperone with PPIase activity, and grp 78 were present within the PSACH chondrocytes but specifically excluded from the enlarged rER inclusions. These results imply that distinct molecular chaperones are associated with the retention of mutant COMP molecules. The induction or up-regulation of various genes encoding molecular chaperones has been shown to occur in response to the accumulation of unfolded proteins within the ER and is known as the unfolded protein response (UPR). It is thought to assist in the refolding of denatured proteins in the ER by increasing the concentrations of chaperones in this compartment and is suggested to be intimately coordinated with the ER-associated degradation pathway (Friedlander et al., 2000; Sidrauski et al., 1998; Travers et al., 2000). The UPR may account for increased levels of HSP47, PDI and calnexin detected within the rER inclusions of PSACH chondrocytes as reported here.

ER-associated degradation of misfolded proteins occurs via ubiquitination and ensuing degradation by the 26S proteasome, as reviewed in (Bonifacino and Weissman, 1998). Proteins normally destined for this degradation pathway are targeted by ubiquitination after their retrotranslocation from the ER (Plemper and Wolf, 1999). We were interested to know whether the mutant COMP molecules in PSACH chondrocytes were being targeted for this ER-associated degradation pathway. Immunolocalization with an antibody to ubiquitin demonstrated only low levels of intracellular staining and ubiquitin was not present within the enlarged rER inclusions. These results imply that the secretory defect in PSACH chondrocytes involves mutant, misfolded COMP molecules that are recognized by specific molecular chaperones but not by ubiquitin. We hypothesize that this is an ER-related event that involves the selective binding and recognition of mutant COMP molecules by specific molecular chaperones preventing its retrotranslocation into the cytosol for ubiqutination and degradation.

A large number of diseases are known in which quality control in the ER plays a role. Many of these involve mutant proteins that are targeted to and retained in the ER and often display a storage phenotype (reviewed in Aridor and Balch, 1999). For example, in α1-antitrypsin deficiency, the mutant secretory glycoprotein is abnormally folded and retained in the ER with apparent hepatotoxic consequences (Perlmutter, 1996). Although PSACH is associated with mutations in the COMP gene it is not known whether the resulting pathology of the disease is a consequence of the ER storage phenotype or a compromised ECM. In considering the overall structure of the COMP molecule, a COMP monomer contains multiple domains that are predicted to fold independently of one another. It is reasonable to assume that the N-terminal α-helical coiled-coil domain of COMP assembles co-translationally as has been shown for TSP1 (Prabakaran et al., 1996). As the chain elongates the independently folding EGF domains also form their appropriate structure, and presumably upon completion of protein synthesis only a small portion

of the COMP molecule (the type 3 domain) is adversely affected by the mutations. If the quality control system recognizes each domain as an independently folded entity, it faces the task of discriminating between six properly folded domains, which accounts for a majority of the protein, and a single misfolded domain which is most likely still associated with molecular chaperones. The resulting oligomeric complex, containing from one to five mutant COMP monomers and associated molecular chaperones, may either be unrecognizable by the ER-associated targeting and degradative machinery, or may be too large to be retrotranslocated through the translocon and out into the cytosol, resulting in prolonged ER retention.

In conclusion, our results suggest impaired secretion of mutant COMP and various ECM molecules causing abnormal accumulations within the rER of PSACH chondrocytes that likely alter normal cell function. This process is specifically mediated by distinct ER chaperones — acting individually or possibly as a complex of proteins — which presumably recognize the misfolded domain of the COMP molecules and play a role in ER retention of the mutant molecules. However, subsequent ER-associated degradation of the mutant molecules does not appear to occur in PSACH. We hypothesize that the selective ER retention of COMP, type IX collagen, low levels of types XI and XII collagen, fibromodulin, decorin and the ER-chaperones HSP47, PDI and calnexin in PSACH leads to impaired cell function and reduced secretion of distinct matrix molecules, which may ultimately lead to a compromised ECM. Further investigations into the different secretory pathways or sorting mechanisms for matrix molecules, ER-associated pathways of folding and degradation, and the co-ordinated involvement of specific molecular chaperones throughout these processes, may provide further insight as to how impaired secretion of mutant COMP ultimately leads to PSACH.

Acknowledgements

This work was supported by grants from Shriners Hospital for Children (to H.P.B., D.R.K., L.Y.S. and W.A.H.) and by Grant AR45582 from the Department of Health and Human Services (to H.P.B).

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