Characterization of a pseudoachondroplasia-associated mutation (His⁵⁸⁷ → Arg) in the C-terminal, collagen-binding domain of cartilage oligomeric matrix protein (COMP)

Luitgard SPITZNAGEL, D. Patric NITSCHKE, Mats PAULSSON, Patrik MAURER and Frank ZAUCKE¹

The Center for Biochemistry, Medical Faculty, University of Cologne, Joseph-Stelzmann-Strasse 52, D-50931 Cologne, Germany

We have introduced a pseudoachondroplasia-associated mutation (His⁵⁸⁷ → Arg) into the C-terminal collagen-binding domain of COMP (cartilage oligomeric matrix protein) and recombinantly expressed the full-length protein as well as truncated fragments in HEK-293 cells. CD spectroscopy revealed only subtle differences in the overall secondary structure of full-length proteins. Interestingly, the mutant COMP did not aggregate in the presence of calcium, as does the wild-type protein. The binding site for collagens was recently mapped to amino acids 579–595 and it was assumed that the His⁵⁸⁷ → Arg mutation influences collagen binding. However full-length mutant COMP bound to collagens I, II and IX, and the binding was not significantly different from that of wild-type COMP. Also a COMP His⁵⁸⁷ → Arg fragment encompassing the calcium-binding repeats and the C-terminal collagen-binding domain bound collagens equally well as the

corresponding wild-type protein. The recombinant fragments encompassing the C-terminal domain alone showed multiple bands following SDS/PAGE, although their theoretical molecular masses could be verified by MS. A temperature-induced conformational change was observed in CD spectroscopy, and negative-staining electron microscopy demonstrated that both wild-type and mutant proteins formed defined elongated aggregates after heating to 60 °C. Our results suggest that the His⁵⁸⁷ → Arg mutation is not itself deleterious to the structure and collagen-binding of COMP.

Key words: calcium, circular dichroism spectroscopy (CD spectroscopy), extracellular matrix, multiple epiphyseal dysplasia, thrombospondin.

INTRODUCTION

COMP (cartilage oligomeric matrix protein) is a secreted homopentameric glycoprotein and belongs to the thrombospondin protein family. It has a typical modular domain structure with a pentamerizing coiled-coil domain at the N-terminus, followed by four epidermal growth factor-like domains, eight calcium-binding thrombospondin type 3 (T3) repeats and a globular domain at the C-terminus [1,2]. When examined by electron microscopy, the five subunits of COMP form a bouquet-like structure with the central assembly domain and five flexible arms terminated by peripheral globular domains [3]. COMP is predominantly found in the extracellular matrix of cartilage, ligaments and tendons [4–6]. Although it has been shown that COMP interacts with collagens I, II and IX in a bivalent cation-dependent manner [7–9], its exact *in vivo* function is still elusive. The interaction with collagens, and the fact that COMP and its proteolytic fragments are released to synovial fluid and serum upon joint degeneration [10,11], suggested a structural role for COMP in the assembly and maintenance of the extracellular matrix. The targeted inactivation of the COMP gene in mice does not result in any skeletal defects [12], but the importance of COMP is underscored by the finding that mutations in the COMP gene result in two related skeletal dysplasias in man, pseudoachondroplasia (PSACH) and multiple epiphyseal dysplasia (MED) [13,14]. The majority of the more than 60 mutations described so far result in single amino acid deletions or substitutions and are localized in the calcium-binding

type 3 repeats, whereas some mutations have been identified in the C-terminal globular domain of COMP [15,16]. More recently, two novel types of mutations have been reported, i.e. one mutation resulting in the deletion of exon 9 and a frame-shift mutation leading to a truncation of the C-terminal domain. The skipping of exon 9 results in a 36-amino-acid in-frame deletion leading to severe PSACH, whereas the phenotypic effect of the frame-shift mutation, which is predicted to produce a protein lacking the 15 C-terminal amino acids, is mild Ribbing-type MED [17].

The mechanisms involved in the pathogenesis of PSACH and MED are currently under investigation. Chondrocytes derived from affected patients are characterized by granular or lamellar inclusions in the endoplasmic reticulum (ER), which contain COMP [18,19]. The vast accumulation of protein within the ER was shown to selectively interfere with secretion of COMP and proteins such as collagens IX, XI and XII, fibromodulin and decorin, but interestingly not with collagen II secretion. Aggrecan was also found in the matrix of patient cartilage [19–21]. Some studies have demonstrated that patient chondrocytes, ligament and tendon cells are still able to secrete mutant COMP [22–23], which might display a dominant-negative effect on extracellular matrix stability and contribute to premature osteoarthritis seen in PSACH and MED patients. In a previous study, we developed a cell-culture model using adenovirally transduced primary chondrocytes and demonstrated that PSACH is, in fact, caused by both intracellular and extracellular pathways [24]. In this model, the secretion of COMP carrying the PSACH-associated D469Δ (Asp⁴⁶⁹ deletion)

Abbreviations used: COMP, cartilage oligomeric matrix protein; EBNA, Epstein–Barr nuclear antigen; ER, endoplasmic reticulum; MALDI-TOF, matrix-assisted laser-desorption ionization–time-of-flight; MED, multiple epiphyseal dysplasia; PNGase F, peptide N-glycosidase F; PSACH, pseudoachondroplasia; rCOMP, recombinant full-length COMP; TBS, Tris-buffered saline; TC, globular C-terminal domain of COMP; T3, thrombospondin type 3 repeats; wt, wild-type.

¹ To whom correspondence should be addressed (e-mail frank.zaucke@uni-koeln.de).

mutation was significantly retarded, but was not completely abolished. The matrix formed by the transduced chondrocytes was severely disturbed, showing only sparse collagen fibrils and amorphous aggregates, in agreement with findings in patient cartilage and cultured patient chondrocytes [9,23]. This could be caused by impaired COMP–collagen interactions, which might affect collagen fibrillogenesis [25]. The second major factor in the pathogenesis of PSACH and MED is presumably a decrease in chondrocyte viability caused by the inclusions [24,26].

Several *in vitro* studies with recombinantly expressed proteins have focused on the analysis of structure and binding properties of mutant COMP. So far, only mutations in the calcium-binding T3 repeats have been studied. The deletion mutation D469 Δ , which is found in 22 % of all PSACH patients, and the substitutions D446N (Asp⁴⁴⁶ $\rightarrow$ Asn) and D361Y (Asp³⁶¹ $\rightarrow$ Tyr) resulted in a decreased calcium-binding capacity. This loss of calcium binding leads to local disturbances in folding of mutant COMP, which has been extensively analysed by spectroscopy [8,27–30]. Differences in the proteolytic susceptibility between wild-type (wt) and mutant COMP provide further evidence that calcium binding is important for the overall COMP conformation [27]. Finally, it has been shown that even the collagen interaction may be influenced by mutations in the calcium binding type 3 repeats, pointing to a long-range intramolecular communication [8]. Rotary shadowing electron microscopy and hydrodynamic studies of mutant COMP indicated that the D469 Δ mutation keeps the protein in an extended conformation, thereby preventing a more compact structure which might be essential for interaction with collagens [27,29]. The crucial role of the C-terminal domain for the COMP–collagen interaction has been demonstrated by electron microscopy [7], and a putative binding site was identified by peptide inhibition [9].

We have now introduced a PSACH-associated mutation into the C-terminal collagen-binding domain of COMP. The selected mutation H587R (His⁵⁸⁷ $\rightarrow$ Arg) is localized within the putative collagen-binding site between residues 579 and 595 [9] and was the first described mutation in exon 16 resulting in typical PSACH ([31]: in this publication the exon was referred to as 19 due to a different nomenclature). We recombinantly expressed full-length and truncated COMP proteins carrying this mutation to characterize the effects of the H587R mutation on the structure and collagen-binding of COMP.

EXPERIMENTAL

Construction of expression vectors for full-length and truncated forms of COMP

The cDNA clone coding for full-length COMP [1] was a gift from Dr Å. Oldberg (Department of Cell and Molecular Biology, Lund University, Lund, Sweden). The recombinant full-length COMP lacking the signal peptide (rCOMP) was described previously [8]. The T3 + TC cDNA (where TC is the globular C-terminal domain of COMP) encoding residues 266–755 was amplified by a 1:2 mixture of *Taq* (PerkinElmer) and *Pfu* (Stratagene) polymerase with primer 1 (5'-GCCCCGTAGCCCGCGACACAGACCTGG-3') and primer M1 (5'-CAATGACTGCGGCCGCTAGGCCCTCCGAG-3'). The cDNA encoding residues 520–755, comprising the TC domain, was generated with primer 2 (5'-GCCCGCTAGCCGAGAACGCCGAGGTAC-3') and primer M1. The PCR products were cloned in either pUC18 linearized with *EcoRV* (rCOMP) or pCR2.1 (T/A, T3 + TC and TC) and finally ligated into an N-terminal His₆-Myc-factor X-tagged eukaryotic expression vector based on pCEP-Pu [32,33]. The cDNA for full-length COMP H587R and T3 + TC H587R was

generated by PCR using primer 3 (5'-GGTTATAGACAAG-ATCGATGTGTGC-3') and mutagenesis primer 5' H587R (5'-GAGGGAACATTCCGTGTAAACACC-3'). A separate reaction was performed with primer M1 and primer 3' H587R (5'-GGTGTTCACGGAATGTTCCCTC-3'). These PCR products served as the template for a final PCR with primer 3 and primer M1 to amplify the C-terminal part (775 bp) of both constructs. The resulting cDNA was ligated into the pCR2.1 (T/A) in *dcm/dam*⁻ *Escherichia coli* SCS110. Parts of the wt sequences (rCOMP wt/T3 + TC wt, in vector pUC18/pCR2.1) were replaced by the corresponding mutated sequence (*ClaI/NotI*). Full-length T3 + TC H587R cDNA was then cloned into the modified expression vector pCEP-Pu. TC H587R cDNA was generated using the combination of primer 2 with mutagenesis primer 5' H587R and primer M1 with mutagenesis primer 3' H587R. An additional silent mutation deleted the restriction site for *AcI* in the mutated constructs to facilitate identification of mutated clones. Correct sequences of all clones were confirmed by sequencing (ABI Prism 377 DNA Sequencer; Applied Biosystems).

Recombinant expression of COMP constructs in HEK-293/EBNA (Epstein–Barr nuclear antigen) cells

For stable episomal expression, 5×10^5 HEK-293/EBNA cells (Invitrogen) were transfected with 1 μ g of purified plasmid by using the non-liposomal reagent FuGENE^{TM} 6 (Roche Diagnostics). The transfected cells were selected with 1 μ g/ml puromycin and grown to confluence in Dulbecco's modified Eagle's medium/Ham's F-12 with 10 % (v/v) foetal calf serum (Life Technologies). Secretion of recombinant proteins was confirmed by SDS/PAGE of samples from transfected and non-transfected cells. Serum-free culture medium was harvested and supernatant containing full-length COMP supplemented with 0.5 mM PMSF and 0.5 mM *N*-ethylmaleimide, whereas T3 + TC supernatant was supplemented with 0.5 mM PMSF only. Cell culture medium was centrifuged at 4000 *g* at 4 °C for 10 min, passed through a paper filter (Schleicher and Schuell, Dassel, Germany), adjusted to pH 8.0 by addition of Tris base and applied to a nickel nitrilotriacetic acid column (Qiagen), previously equilibrated with 0.3 M NaCl in 50 mM NaH₂PO₄, pH 8.0, containing protease inhibitors. Elution was performed with a gradient of 0–0.25 M imidazole in the same buffer. COMP-containing fractions were pooled and subjected to gel filtration on Sepharose CL-6B (Amersham Biosciences), equilibrated in 50 mM NaCl, 0.5 mM PMSF and 50 mM Tris/HCl, pH 8.6. For the T3 + TC and TC proteins, final purification was achieved by ion-exchange chromatography on a MonoQ column (Amersham Biosciences) equilibrated in 50 mM Tris/HCl, pH 8.6, and proteins were eluted with a gradient of 0–1 M NaCl in the same buffer. Concentration of the purified protein was performed with disposable ultrafiltration device (Vivascience, Lincoln, U.K.). The final preparation was dialysed against either Tris-buffered saline (TBS; 0.15 M NaCl and 50 mM Tris/HCl, pH 7.4), or 5 mM Tris/HCl, pH 7.4, and aliquots were stored at –20 °C. The purity and size of all proteins were assessed by SDS/PAGE, with and without prior reduction of disulphide bonds.

PNase F (peptide N-glycosidase F) digestion and MALDI-TOF (matrix-assisted laser-desorption ionization–time-of-flight) MS

Protein (8 μ g) in TBS was mixed with 0.1 % (w/v) SDS and incubated for 15 min at 100 °C. After cooling to room temperature (22 $\pm$ 1 °C), 0.5 % (v/v) Nonidet P40 and 5 units of PNase F (Roche Diagnostics) were added, and the samples were incubated

over night at 37 °C. Detergents were removed by ethanol precipitation and proteins were resuspended in 5 µl of TBS. Equal aliquots of digested and undigested protein were analysed by MALDI-TOF MS. The samples were prepared as described [34] with α -cyano-4-hydroxycinnamic acid (Sigma) as the matrix. Detection and analysis of cations was performed utilizing the high or low mass detector in the linear mode of a Reflex III mass spectrometer (Bruker). The time-of-flight analyser was calibrated using the M_r of recombinant protein A (RepliGen, Waltham, MA, U.S.A.) of 44 610.3 and BSA dimer (Sigma) of 132 859.0.

CD spectroscopy

CD spectra in the far UV region were recorded in a thermostatically controlled quartz cell of 1 mm optical path length in a JASCO 715 CD spectropolarimeter. Spectra were measured with the proteins dissolved in 5 mM Tris/HCl, pH 7.4, and after the addition of <0.5 mM calcium chloride. Reversal of the conformational change was examined after addition of 0.5 mM EDTA. Five complete spectra (190–250 nm) were accumulated to improve the signal/noise ratio and buffer spectra were subtracted. Spectra of the temperature gradient were recorded once at 215 nm from 20 to 80 °C.

Electron microscopy

During the CD measurements, aliquots of wt COMP and the H587R mutant were taken at 20 and 60 °C. The proteins were immediately adsorbed onto carbon-coated grids that had been rendered hydrophilic by glow discharge in air. After negative staining with 0.75 % (w/v) uranyl formate [35], the samples were subjected to electron microscopy in a Zeiss EM 902-A electron microscope (acceleration voltage, 80 kV).

Solid-phase ligand-binding assays

Collagen I was purchased from Sigma, collagen II was a gift from Dr U. Hansen (Institute of Physiological Chemistry and Pathophysiology, University of Münster, Münster, Germany), and recombinant collagen IX from Dr T. Pihlajamaa (NMR Laboratory, University of Helsinki, Helsinki, Finland) and Dr L. Ala-Kokko (Center for Gene Therapy, Tulane University Health Sciences Center, New Orleans, LA, U.S.A.). Collagen I had been isolated after pepsin digestion from bovine dermal skin, collagen II was extracted and chromatographically purified from chicken chondrocytes, and collagen IX was produced as described previously [36]. Each collagen was dissolved in 0.5 mM ethanoic acid. The T3 + TC and TC proteins were biotinylated before performing the binding assay. Samples were dialysed overnight at 4 °C against 10 mM borate, pH 8.8, in 0.15 M NaCl and 10 % freshly prepared *N*-hydroxysuccinimidobiotin (1 mg/ml in DMSO, Calbiochem) was added for 4 h at 4 °C. The reaction was stopped by addition of (NH₄)₂SO₄ to 10 mM and the biotinylated proteins were dialysed against TBS. Ligand-binding studies were performed as solid-phase assays in 96-well plates (Maxisorb, Nunc, Wiesbaden, Germany) with 50 µl of collagen solution (2.5 µg/ml in 0.18 M ethanoic acid) used to coat each well overnight at room temperature. After washing with 0.05 % (v/v) Tween 20 and 0.5 mM ZnCl₂ in TBS, the wells were blocked for 2 h with 200 µl of 10 mg/ml BSA in TBS at room temperature. After renewed washing, the wells were incubated for 1 h at room temperature with ligands in TBS containing 0.5 mM ZnCl₂. For the determination of affinity, the ligands were used at different concentrations. Non-specific binding was determined at each

Figure 1 Domain composition of COMP and its recombinant fragments

tag, His₆-myc-factor X tag; PD, pentamerization domain at the N-terminus; EGF, epidermal growth factor-like domains; asterisk (*) calcium-binding EGF-like domains. Eight T3 repeats are shown as rectangles. The position of the PSACH-causing mutation H587R is indicated.

ligand concentration in albumin-blocked wells lacking a collagen coat. Bound COMP was detected either with a monoclonal antibody against the myc-epitope (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), followed by peroxidase-coupled rabbit anti-mouse IgG (Dako Diagnostika GmbH, Hamburg, Germany), or by a peroxidase-coupled streptavidin. The absorbance was recorded at 450 nm using 3,3',5,5'-tetramethylbenzidine as a chromogenic substrate.

RESULTS

Full-length and truncated (T3 + TC and TC alone) COMP were expressed in a eukaryotic expression system. The PSACH-associated point mutation (A1785G) was introduced by PCR, which resulted in the change of His⁵⁸⁷ to Arg (H587R; Figure 1). This point mutation causes severe PSACH [31].

Characteristics of H587R mutant COMP molecules expressed in HEK-293 cells

Mutant and wt proteins were expressed in HEK-293 cells, purified from the serum-free cell culture supernatants and the final preparations were analysed by SDS/PAGE in the absence and presence of a reducing agent (Figure 2). Under non-reducing conditions, wt and mutant (H587R) full-length COMP migrated as pentamers of about 550 kDa. As previously shown, rCOMP is partially cleaved by a protease in cell culture leading to the appearance of additional bands during purification [8]. The protease cleaves at position 77–78 between the coiled-coil pentamerization domain and the C-terminal arm of COMP of approx. 100 kDa. This results in molecules with a pentameric coiled coil carrying one to five COMP arms. With the exception of the four-armed COMP, the smaller cleavage products could be removed from the pentameric fraction by gel filtration. Under reducing conditions, a single band was observed (Figure 2B), since the recombinant COMP carried an N-terminal tag and the C-terminal cleavage products were lost during affinity chromatography and gel filtration. The fragments encompassing the T3 + TC domains yielded single bands under both non-reducing and reducing conditions. The different migration under these two conditions is presumably due to the presence of intramolecular disulphide bonds. The mass was confirmed by MALDI-TOF MS and corresponded to the expected theoretical molecular mass of 60.0 kDa (results not shown). The isolated TC domain gave multiple bands

Figure 2 SDS/PAGE of rCOMP and fragments thereof

Recombinant proteins were separated on polyacrylamide gels under non-reducing (**A**) and reducing conditions (**B**) and stained with Coomassie Brilliant Blue. Lanes were loaded with full-length wt COMP (lanes 1), full-length COMP carrying the H587R mutation (lanes 2), wt (lanes 3) and mutant (lanes 4) T3 + TC protein, and wt (lanes 5) and mutant (lanes 6) TC domain. The migration position of standard proteins is given in kDa. Arrows indicate the origin and the front of the gel. Note that some samples were run on different gels with slightly different percentages of polyacrylamide and therefore the migration behaviour can not be directly compared.

which differed in their migration behaviour during SDS/PAGE and native PAGE (results not shown). Immunoblot analysis confirmed that all bands consisted of COMP. Degradation at the N-terminus could be excluded, since all TC domain bands were detected with an antibody directed against the N-terminal tag. To analyse a potential degradation at the C-terminus, all three bands were cut out of the gel and were separately digested with *Staphylococcus aureus* V8 protease. In digests of all bands, the very C-terminal peptide could be detected by MALDI-TOF MS, thereby excluding a C-terminal degradation. Furthermore, MALDI-TOF analysis of the undigested recombinant protein revealed a sharp, single peak of 33.9 kDa for the wt TC domain (Figure 3). PNGase F digestion induced a complete shift to 32.2 kDa, which exactly matches the calculated theoretical molecular mass. The difference of 1.7 kDa probably represents a single oligosaccharide, in good agreement with previous results on the post-translational modifications in COMP [37]. The additional peak at 34.6 kDa is due to the PNGase F itself.

The mutant TC domain appeared in two forms before deglycosylation, with a 32.3 kDa species corresponding to the non-glycosylated form and a 34.0 kDa glycosylated form. This suggests that the mutation affects the recognition by glycosyl-transferases, presumably due to a local conformational heterogeneity.

H587R mutation does not cause drastic conformational changes

Folding of full-length wt and mutant COMP was investigated by CD spectroscopy (Figure 4). As previously shown for PSACH-associated mutations in the T3 repeats [8], the H587R mutation

Figure 3 MALDI-TOF MS of the COMP TC domain

The masses of wt (**A**, **C**) and mutant TC domain (**B**, **D**) before (**A**, **B**) and after (**C**, **D**) digestion with PNGase F were determined. a.i., arbitrary intensity.

has, if any, only subtle effects on the secondary structure of the full-length protein. The marked negative ellipticity at 200 nm and low negative values at 220 nm indicate a high content of random coil and relatively little α -helical structure in the full-length protein. Addition of 0.2 mM calcium chloride induced a slight conformational change. Increasing the calcium concentrations further led to a loss of ellipticity due to aggregation, which could not be reversed by addition of EDTA. Interestingly, the mutant protein was completely insensitive to the addition of calcium and did not aggregate even at 0.5 mM calcium or after addition of EDTA.

CD analysis of the truncated constructs T3 + TC and TC also did not reveal major differences caused by the H587R mutation (Figure 5). The T3 + TC construct showed a similar secondary structure to full-length COMP with a high proportion of random structures and some α -helix. The TC domain alone showed a minimum at 210 nm, indicative of a structure rich in β -sheets. One possible explanation for the multiple bands seen with the TC protein is that it occurs as different folding intermediates even in the presence of SDS. Therefore we recorded CD spectroscopy melting curves at a fixed wavelength of 215 nm to identify potential transition states (Figures 5C and 5F). The T3 + TC construct did not show any significant conformational change upon heating to 60 °C, whereas the TC domain changed its conformation biphasically. In a first phase with a transition temperature of approx. 45 °C, the ellipticity increased, whereas at temperatures above 60 °C, the ellipticity decreased. The second phase was parallel with precipitation of the protein.

The decreased ellipticity seen with both the wt and the mutant TC domain at elevated temperatures suggested an increase in structure. We therefore analysed the shape and the state of aggregation of the proteins by negative-staining electron microscopy (Figure 6). At 20 °C, single globular structures could be seen for both the wt and the mutant COMP. The increase in temperature up to 60 °C induced an association of monomers resulting in a well-defined chain-like arrangement

Figure 4 Calcium-induced conformational changes in full-length wt COMP and the full-length H587R mutant

CD spectra of wt (solid lines) and mutant COMP (dotted lines) were recorded in the absence of calcium (A), with increasing calcium concentrations as indicated (B), (C) and (D) and after addition of 0.5 mM EDTA (E).

of globular domains. Both the wt and the mutant proteins gave rise to indistinguishable structures consisting of three to four globules.

The H587R mutation does not dramatically affect collagen binding

To assess function, we investigated the binding of each of the COMP proteins to collagens I, II and IX in solid-phase binding assays. Collagens were coated on microtitre plates and were incubated with increasing concentrations of COMP in the presence of 0.5 mM zinc. In each case, the binding was concentration-dependent and saturable (Figure 7) and concentrations needed for half-maximal saturation could be determined (Table 1). The values obtained for the interaction of full-length COMP proteins with all tested collagens were in the lower nanomolar range, in good agreement with previous studies [7,8]. Due to the monomeric structure of the truncated T3 + TC protein, the molar concentrations needed for half-saturation with these fragments were higher than for the pentameric full-length protein, but still in the nanomolar range. In neither the full-length COMP nor the T3 + TC proteins did the H587R mutation lead to significant differences in the binding to collagens, although the apparent dissociation constants for the mutated forms indicate a slightly lower affinity. Interestingly, the isolated wt TC domain shows a marked loss of collagen affinity as compared with the larger COMP proteins, whereas the values for the mutant TC domains were in a similar range as those for the T3 + TC protein.

DISCUSSION

More than 60 mutations in the gene coding for COMP have been described, leading to either PSACH or MED [15,16]. The mutations include single amino acid exchanges, small deletions, insertions and, more recently identified, a deletion of exon 9 and a truncation of the 15 C-terminal amino acids [17]. The mutations are localized in different functional domains of the protein, but result in a similar phenotype in affected patients. This suggests a common mechanism in the pathogenesis of PSACH caused by mutations in the T3 repeats, even though both intra- and extracellular pathways seem to contribute [24]. In previous studies, only mutations in the T3 repeats were characterized at the protein level and underscored the importance of the calcium-binding domain for the structure and function of COMP. In addition, it has been shown that mutations in the T3 repeats exert a long-range effect on collagen binding mediated by the C-terminal part of the molecule [8]. A putative binding site for collagens between residues 579 and 595 in the C-terminal domain of COMP was identified by peptide-inhibition assays [9]. Within this region, four disease-causing mutations have been identified, affecting three closely clustered amino acid residues, E583K (Glu⁵⁸³ → Lys), T585M (Thr⁵⁸⁵ → Met), T585R (Thr⁵⁸⁵ → Arg) and H587R [31,38,39].

In the present study, we introduced the PSACH-causing mutation H587R into the COMP cDNA and expressed recombinant full-length mutant COMP. To address the local effects of the amino acid exchange, we also produced T3 + TC and TC fragments carrying this mutation. Both wt and mutant full-length proteins were secreted as pentamers, showing that the mutation in the C-terminus does not interfere with assembly at the N-terminal coiled-coil domain. There were no signs of intracellular retention and/or accumulation, confirming previous results for the secretion of other MED- and PSACH-causing mutant COMP variants by HEK-293 cells [8]. However, these findings are in contrast with observations in cultures of patient chondrocytes [19,26] or bovine articular chondrocytes, which had been transduced with adenoviruses encoding the D469Δ mutant [24], showing that mutated proteins are retained intracellularly. The HEK-293 cell system is therefore not suited for studies of

Figure 5 CD spectra of truncated COMP proteins

CD spectra for T3 + TC (A)–(C) and TC domains (D)–(F) were recorded at 20 °C (A), (D), 60 °C (B), (E) and with increasing temperature at a fixed wavelength of 215 nm (C), (F). Solid lines represent the wt proteins, dotted lines show the corresponding mutant forms.

Figure 6 Negative-staining electron microscopy of the COMP TC domain

Overviews of the wt TC domain (A) and the H587R mutant (C) at 20 °C, and wt (B) and mutant TC domains (D) after heating to 60 °C. On the right-hand side, the directly adjacent smaller panels show selected particles magnified from (B) and (D) respectively. The bar represents 100 nm in the overviews and 25 nm in the panel of selected particles.

Figure 7 Binding of COMP constructs to collagens I, II and IX in an ELISA-style solid-phase assay

Collagen I (A), (D) and (G), II (B), (E) and (H), and IX (C), (F) and (I) were coated overnight at 2.5 $\mu\text{g/ml}$ onto plastic wells and incubated with 0–48 $\mu\text{g/ml}$ COMP proteins in TBS with 0.5 mM ZnCl_2 for 1 h. This corresponds to 0–87 nM for full-length (A)–(C), 0–800 nM for T3 + TC (D)–(F) and 0–1400 nM for TC COMP (G)–(I) respectively. Solid lines with closed circles represent the wt proteins, dotted lines with open circles the corresponding mutant forms. Bound COMP was detected using an antiserum against the myc epitope followed by incubation with a peroxidase-coupled antiserum against mouse IgG or peroxidase-coupled streptavidin using 3,3',5,5'-tetramethylbenzidine as a chromogenic substrate and recording absorbance at 450 nm. Results are means $\pm$ S.D. of triplicate determinations.

Table 1 Binding of wt and H587R mutant COMP to collagens I, II and IX

Concentrations needed for half-maximal saturation of the binding of wt and corresponding mutant COMP proteins to collagens I, II and IX were evaluated from binding measurements using ELISA-style binding assays in the presence of 0.5 mM zinc. Values are in nM.

Collagen	rCOMP		T3 + TC		TC	
	wt	H587R	wt	H587R	wt	H587R
I	3.9	8.5	29.7	48.5	274.1	29.0
II	5.2	35.5	105.5	140.1	4942.8	96.9
IX	6.3	11.2	70.6	127.5	349.5	211.4

the pathological intracellular trafficking of COMP, but our results demonstrate that the H587R mutation is not itself sufficient to cause intracellular accumulation of mutant COMP.

The H587R mutation has only little influence on the overall secondary structure of COMP, as analysed by CD spectroscopy, and is in this regard similar to two mutations in the T3 repeats (D361Y, D469 Δ), which have been well characterized [8,27]. Nonetheless, local conformational changes were observed for the mutants by electron microscopy, sedimentation analysis, fluorescence and NMR spectroscopy of smaller fragments [8,27,29,30]. We therefore investigated shorter fragments of COMP, the T3 + TC construct and the TC domain alone. As seen for full-length COMP, no differences in expression and CD spectra could be observed for the wt and mutant T3 + TC domain. Also in thermal denaturation behaviour, differences were not discerned. This extends the previous observations for PSACH and MED mutations in the T3 repeats, which showed that, in addition to mutant COMP, chondrocyte-specific factors are needed to cause the inclusions.

The TC domain can be identified by sequence homology and is attributed to the globular structure at the end of the COMP arms. Nonetheless, its independent folding is a matter of debate. For thrombospondin-2, the TC domain alone was not secreted in a eukaryotic expression system, whereas a construct encompassing T3 repeats and the C-terminal domain was produced in high amounts [40,41]. In contrast, Holden et al. [9] and the present study demonstrate that the TC domain of COMP can be expressed independently of the T3 repeats. This could be related to a different disulphide pattern between thrombospondin-2 and COMP. The T3 + TC construct of thrombospondin-2 contains 18 cysteine residues, all of which participate in disulphide bonds. A cysteine residue in the C-terminal domain thereby forms a disulphide bond with a cysteine residue attributed to the T3 repeats [41]. The T3 + TC construct of COMP contains 19 cysteine residues and a sequence alignment shows that the additional cysteine residue is located in the C-terminal domain. It remains to be determined whether a disulphide bond between the T3 repeats and the TC domain is present in COMP.

The secondary structure of the TC domain was analysed using the PSIPRED protein structure prediction server [42]. The TC domain is predicted to contain 41 % β -strands and 9 % α -helices. These numbers are in good agreement with secondary-structure elements calculated from CD spectra using the SELCON program [43], yielding 34.9 % β -strands and 9 % α -helices for the wt protein, and 31.3 % β -strands and 7 % α -helices for the mutant protein. These results confirm that the TC domain is dominated by β -structures. However, the TC domain did not behave as a stable domain. Multiple bands are seen in SDS/PAGE and native gels, in spite of the uniform molecular mass determined by MALDI-TOF MS. These bands cannot be due to disulphide shuffling, since the C-terminal domain contains only two cysteine residues. The temperature-induced association and the impaired collagen binding of the TC domain, but not of the T3 + TC construct, indicates that the isolated TC domain might not reflect the same structural and thus functional characteristics as if it is embedded in full-length COMP. TC domains that formed monomeric globular structures at room temperature assembled at 60 °C into well-defined fibrillar structures. Similar conformational changes have also been observed for phosphoglycerate kinase, which forms fibrillar structures in the form of small oligomers and even larger protofibrils. Acquisition of a β -sheet structure occurs concomitantly with oligomer formation [44]. Heat-induced association has also been described for β -lactoglobulin. It also forms β -sheets and the intermediates of the heat-induced association show typical molten globule characteristics [45]. Interestingly, the introduction of the H587R mutation into the TC domain induced neither a change in secondary structure at ambient temperatures nor a change in the temperature-dependence of the CD melting curves. Fibrillar structures indistinguishable from the ones formed by wt TC domains were observed at 60 °C. Although these observations should not be overinterpreted, it indicates that the H587R mutation does not dramatically influence the conformation of the TC domain.

Fibrillar collagens I, II and IX bind to COMP and electron micrographs revealed that the C-terminal globular TC domain contains the binding sites for collagens [7–9]. This was confirmed by the finding that recombinant T3 + TC constructs bound to the NC4 domain of collagen IX (T. Pihlajamaa, H. Lankinen, J. Ylöstalo, L. Valmu, J. Jäälinoja, F. Zaucke, L. Spitznagel, S. Gössling, A. Puustinen, J. Peränen, P. Maurer, L. Ala-Kokko and I. Kilpeläinen, unpublished work). Using a peptide-inhibition assay, the collagen-binding site was mapped to amino acids 575–594 within the TC domain [9]. Since different mutations

causing PSACH or MED are present within this region, we had expected that these mutations would interfere with collagen binding. However, the H587R mutation did not show a significant effect on the binding to collagens I, II and IX when present in full-length COMP and the T3 + TC constructs. Only small 2- to 7-fold decreases in apparent affinity were observed. This questions the importance of H587R for collagen binding and the exact binding site on COMP remains to be clearly identified.

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