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COMP Mutations: Domain-Dependent Relationship Between Abnormal Chondrocyte Trafficking and Clinical PSACH and MED Phenotypes

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Abstract Mutations in cartilage oligomeric matrix protein (COMP) produce clinical phenotypes ranging from the severe end of the spectrum, pseudoachondroplasia (PSACH), which is a dwarfing condition, to a mild condition, multiple epiphyseal dysplasia (MED). Patient chondrocytes have a unique morphology characterized by distended rER cisternae containing lamellar deposits of COMP and other extracellular matrix proteins. It has been difficult to determine why different mutations give rise to variable clinical phenotypes. Using our in vitro cell system, we previously demonstrated that the most common PSACH mutation, D469del, severely impedes trafficking of COMP and type IX collagen in chondrocytic cells, consistent with observations from patient cells. Here, we hypothesize that PSACH and MED mutations variably affect the cellular trafficking behavior of COMP and that the extent of defective trafficking correlates with clinical phenotype. Twelve different recombinant COMP mutations were expressed in rat chondrosarcoma cells and the percent cells with ER-retained COMP was assessed. For mutations in type 3 (T3) repeats, trafficking defects correlated with clinical phenotype; PSACH mutations had more cells retaining mutant COMP, while MED mutations had fewer. In contrast, the cellular trafficking pattern observed for mutations in the C-terminal globular domain (CTD) was not predictive of clinical phenotype. The results demonstrate that different COMP mutations in the T3 repeat domain have variable effects on intracellular transport, which correlate with clinical severity, while CTD mutations do not show such a correlation. These findings suggest that other unidentified factors contribute to the effect of the CTD mutations. J. Cell. Biochem. 103: 778–787, 2008. © 2007 Wiley-Liss, Inc.

Key words: COMP; pseudoachondroplasia; (PSACH); multiple epiphyseal dysplasia; (MED); chondrocyte; endoplasmic reticulum

COMP is a large extracellular glycoprotein initially discovered in cartilage but subsequently found in a number of other tissues [Hedbom et al., 1992; DiCesare et al., 1994; Hecht et al., 1998]. It is a pentameric protein and the fifth member of the thrombospondin gene family [Adams et al., 1995]. The specific function of COMP is unknown, but other thrombospondins have adhesive properties [Adams, 2001]. Recent work suggests that COMP may provide an interface in the cartilage extracellular matrix by mediating interactions between cartilage collagen fibrils and interfibrillar constituents [Budde et al., 2005] and may stimulate chondrocyte proliferation through a direct interaction with granulin-epithelin precursor [Xu et al., 2007].

Mutations in COMP result in two distinct autosomal dominant skeletal dysplasias, PSACH and MED [Briggs et al., 1995; Hecht et al., 1995; Briggs et al., 1998; Unger and Hecht, 2001]. PSACH is a dwarfing condition associated with shortening of the tubular bones, abnormal joints and early onset

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osteoarthritis that necessitates hip replacement in early adulthood [Hall, 1975; McKeand et al., 1996]. MED is an allelic condition in which stature is minimally reduced but hips are abnormal and easily eroded, often requiring replacement in early adulthood as well [Unger and Hecht, 2001; Unger et al., 2001]. MED is genetically heterogeneous and is caused by mutations in COMP (EDM1), matrilin-3 (EDM5) or any of the three chains of type IX collagen (EDM2, 3, 6). In this paper, we address only MEDs resulting from COMP mutations (EDM1). Although a certain degree of phenotypic variability is associated with COMP mutations, based on clinical and radiographic findings, individuals who do not exhibit dwarfism are classified as MED, whereas, PSACH individuals are disproportionately short with heights ranging from 3′ to 4′9″ [Hall, 1975; McKeand et al., 1996; Spranger et al., 2002].

More than 60 novel COMP mutations have been identified among PSACH and MED patients. The majority are found in the highly conserved type 3 repeat (T3) domains [Unger and Hecht, 2001; Kennedy et al., 2005a], suggesting the importance of this region and its critical role in protein conformation. By comparison, only 10 mutations have been identified outside T3 domains, and these are clustered in the CTD [Kennedy et al., 2005b].

Mutations in COMP cause a cellular phenotype characterized by large rER cisternae that are filled with lamellar depositions of COMP and several other cartilage extracellular matrix proteins (specifically, type IX collagen and matrilin-3), which form a unique intralumenal matrix network [Cooper et al., 1973; Hecht et al., 2001, 2004]. Previously we showed that the most common COMP mutation, D469del, which causes PSACH, retarded the ER exit of COMP when expressed in rat chondrosarcoma (RCS) chondrocytic cells, but allowed normal trafficking through the secretory pathway in non-chondrocytic COS-1 cells [Chen et al., 2004]. In this study, we tested the hypothesis that specific COMP mutations differentially affect the extent of defective trafficking of mutant COMP in a manner that correlates with the clinical phenotype. We selected 11 additional COMP mutations, as well as the common D469del mutation, that cause the range of mild (MED) to severe (PSACH) clinical symptoms. Using our in vitro RCS cell model system [Chen et al., 2004], we show that the cellular trafficking phenotype exhibited by specific mutant COMPs is predictive for COMP mutations found in the calcium binding T3 repeats, but not for mutations in the CTD.

MATERIALS AND METHODS

Construction of COMP Mutations

The initial construct consisted of a CMV promoter driving the open reading frame of WT-COMP with a C-terminal FLAG epitope [Chen et al., 2004]. The native signal peptide was used in these experiments. COMP mutation constructs were generated using the QuickChange Site-Directed Mutagenesis Kit (Qiagen) with the following modifications. A mutagenic primer and a primer approximately 100–200 bp away were used to amplify a mutant fragment using high fidelity polymerase, platinum Taq (Invitrogen) and standard PCR methods. The primers used to construct the COMP mutations are shown in Table I. The mutant fragments were purified and used to amplify the full constructs. Approximately 20–50 ng of each mutagenic fragment was combined

TABLE I. Primers Used to Generate COMP Mutations

Mutation	Change	Mutant primer	Second primer
D271H	gac → cac	TCCTCTGTGGTCGCGACACT**c**ACCTAGACGGCTTCCCGGACG	A
L272P	cta → cca	CTCTGTGGTCGCGACACTGACC**c**AGACGGCTTCCCGGACG	A
P276R	cgg → ccg	CACTGACCTAGACGGCTTC**c**GGGACGAGAAGCTGCGCTGCCCGG	A
G299R	ggg → agg	GCGTGACTGTGCCCAACTCA**a**GGCAGGAGGATGTGGACCGCG	A
D302V	gat → gtt	GTGCCCAACTCAGGGCAGGAGG**t**TGTGGACCGCGATGGCATCGGAG	A
G309R	gga → aga	GATGTGGACCGCGATGGCATC**a**GAGACGCCTGCGATCCGGATG	A
D473G	gac → ggc	GATGCCTGCGACGACGACGACG**g**CAATGACGGAGTCCCTGAC	B
D473N	gac → aac	GATGCCTGCGACGACGACGAC**a**ACAATGACGGAGTCCCTGAC	B
T585M	acg → atg	GGCGTGGACTTCGAGGGCA**t**GTTCCATGTGAACACGGTC	C
T585R	acg → agg	GGCGTGGACTTCGAGGGCA**g**GTTCCATGTGAACACGGTC	C
H587R	cat → cgt	GCTGGACTTCGAGGGCAC**g**TTCCGTGTGAACACGGTCACGGATGACG	C

Primer A: GTACCCTAGGGCAGTTGTCGGCCTGG; Primer B: GCACCACCCAGTTGGGGTCAATCTGCG; Primer C: CGGTGCTCAACCAGGGAAGGGAG.

with 50 ng of the WT COMP-FLAG plasmid and kit contents. This mixture was cycled between 95 and 68°C (95°C for 1 min to denature the DNA template before cycling, 95°C for 50 s then 68°C for 24 min for 18 cycles, with a final 68°C for 7 min for a final extension step). Bacterial colonies containing the mutated constructs were obtained, DNA extracted, and constructs confirmed by DNA sequencing.

Cell Culture, Transfection, Immunofluorescence Localization and Microscopy

The chondrocytic Rx RCS cell line [King and Kimura, 2003] was used for cell culture and transfection as previously described [Chen et al., 2004]. After culture for 17–20 h, the transfected cells were fixed in methanol at −20°C and used for immunofluorescence staining. Fixed cells were re-equilibrated in phosphate-buffered saline (PBS) prior to antibody incubations. Primary antibody incubations were performed at 37°C for 1–2 h followed, after extensive washes in PBS, by incubating in fluorophore-conjugated secondary antibody IgGs (Jackson ImmunoResearch Laboratories) for 1 h at 37°C. Expressed COMP proteins were detected with the M2 mouse monoclonal anti-FLAG antibody (Eastman Kodak) (final concentration 0.03 mg/ml). Targeting to the Golgi complex was verified in co-localization experiments by following the above incubations with sequential incubations in rabbit antibodies to the Golgi compartment specific marker, TGN38, and Texas Red-labeled secondary antibody IgGs. The specimens were analyzed and images acquired using a Leica DMRB microscope equipped with a Hamamatsu CCD camera driven by the Openlab imaging program (Improvision).

For quantitative analysis of cell populations, cells were scored according to the presence or absence of anti-FLAG immunostaining for expressed COMP in the Golgi complex. Cells were assigned to one of two categories, either having expressed COMP detected in the Golgi complex, or having no expressed COMP, or trace amounts of expressed COMP, present in the Golgi complex. For each construct, we analyzed a total of at least 300 expressing cells pooled from three independent transfections, using two separate staining reactions for each independent transfection. Although the rates of transfection sometimes varied among different experiments and for different constructs, we found that the percentage of cells with Golgi-localized, expressed COMP was not significantly affected by transfection efficiencies.

RESULTS

Selection of COMP Mutations and Overview of Clinical Phenotypes

Mutations from the calcium-binding motif of the linker (between the T2 and T3 repeats) and T3 repeats and other mutations from the CTD of COMP were selected as representatives of the mild to severe spectrum of clinical phenotypes observed in MED and PSACH, respectively (Table I, Fig. 1). All of the published reports were reviewed to evaluate the clinical data of patients classified as PSACH or MED. These diagnoses were based on the accepted diagnostic criteria for these disorders [Spranger et al., 2002; Superti-Furga et al., 2007]. Two mutations, G299R and T585M, were classified as mild PSACH [Briggs et al., 1998; Ikegawa et al., 1998]. Review of the published clinical data indicates that the individual with the G299R mutation meets the clinical criteria for PSACH [Ikegawa et al., 1998]. The physical findings of the T585M patient and affected family members are most consistent with MED, the diagnosis also reported by Czarny-Ratajczak et al. [2001] for this mutation [Briggs et al., 1998; Czarny-Ratajczak et al., 2001]. The clinical phenotypes and corresponding references are included in Table II.

PSACH and MED mutations were selected at residues 271–289 (Linker T2–T3), residues 290–309 (T3) and residues 585–587 (CTD). Calcium binding motifs are located in both the linker region and the T3 repeats. Mutations in the linker were chosen to determine if disrupting calcium binding in this region causes the same trafficking defect as disturbing calcium binding in the T3 repeats. The three mutations selected from the linker region include two PSACH mutations (D271H and L272P) and one MED mutation (P276R). Five mutations in the T3 repeats were chosen. The most common PSACH mutation, D469del, characterized in the previous study [Chen et al., 2004], was included as a reference standard. Three mutations (G309R-PSACH, G299R-PSACH, and D302V-MED) are clustered in the same T3 domain and reflect the spectrum of COMP-related disorders. D473G and D473N, two PSACH mutations, were chosen to assess

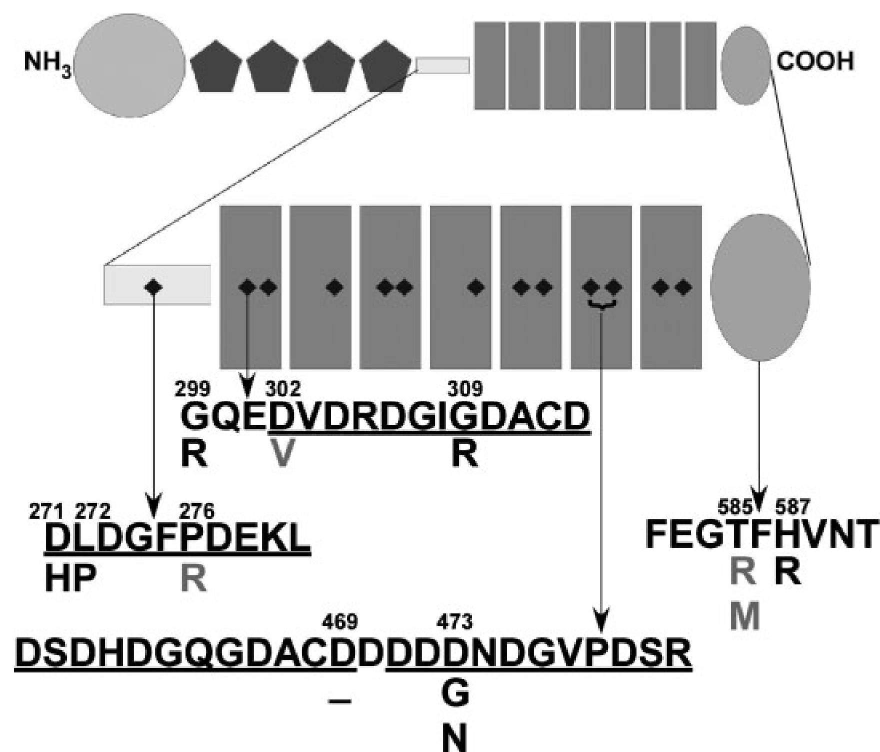

Fig. 1. Mutations of COMP used in this study. A schematic of the domains of COMP is shown. The circle represents the N-terminal pentamer-forming domain (exons A–C), the four grey pentagons represent the EGF-like domains, also known as T2 (exons 11–13B), the white rectangle represents the linker region between T2 and T3 repeats, the seven grey rectangles represent T3 repeats (exons 13C–18A), which are the major calcium-binding domains and lastly, the oval represents the CTD (exons 18B–22). Below the COMP diagram, the T3 and CTD regions are enlarged. Small black diamonds represent the calcium-binding motif, DXDXDXXXDXXD, and these motifs are underlined in the amino acid sequences, which are shown below the diagram, and are also indicated by the black arrows. Mutations are shown below the amino acid sequence; black letters represent PSACH mutations (– for deletion at amino acid 469) and grey letters are for MED mutations. The N and C termini are indicated.

mutations at the same residue. Mutations in the CTD, the region proposed to interact with other ECM constituents, were selected for comparison. The T585M and T585R mutations in the CTD were categorized as MED, while the H587R mutation, two residues closer to the C-terminus, causes PSACH [Briggs et al., 1998; Czarny-Ratajczak et al., 2001]. In total, eleven new mutations were selected for study and the twelfth mutation, D469del, was included as the previously characterized, standard PSACH reference mutation. WT COMP was used as the normal control.

Defective Intracellular Trafficking Behavior of Mutant COMP in Chondrocytic RCS Cells Correlates With Clinical Phenotypes of PSACH and MED

Previously, we reported a cell culture model for PSACH employing RCS cells in which a

TABLE II. COMP Mutations and Corresponding Phenotype

Mutation	Domain	Diagnosis	Ref
D271H	Linker T2–T3	PSACH	Deere et al. [1999]
L272P	Linker T2–T3	PSACH	Deere et al. [1999]
P276R	Linker T2–T3	MED	Czarny-Ratajczak et al. [2001]
G299R	T3	PSACH	Ikegawa et al. [1998]
D302V	T3	MED	Deere et al. [1999]
G309R	T3	PSACH	Delot et al. [1998]
D469del	T3	PSACH	Briggs et al. [1995], Hecht et al. [1995]
D473G	T3	PSACH	Ikegawa et al. [1998]
D473N	T3	PSACH	Deere et al. [1998]
T585M	CTD	MED	Briggs et al. [1998]
T585R	CTD	MED	Briggs et al. [1998], Kennedy et al. [2005b]
H587R	CTD	PSACH	Deere et al. [1999]

chondrocyte-specific intracellular trafficking defect was demonstrated; defective trafficking was observed for D469del mutant COMP when it was expressed in the chondrocytic RCS cells, but not when expressed in non-chondrocytic cells [Chen et al., 2004]. WT COMP was localized in the juxtanuclear Golgi region of the RCS cells, with some protein in the ER (as shown in Fig. 2A,B). In a manner reflecting the severe PSACH phenotype, the D469del mutant COMP was retained in the ER and absent from the juxtanuclear Golgi region. The trafficking defect of the D469del mutant was confirmed by Western blot analysis of immunoprecipitated cell lysate and medium from chondrocytes expressing WT COMP and the D469del mutant [Chen et al., 2004].

Here, we used the RCS model system to further define the relationship between mutated COMP and defective intracellular trafficking behavior. COMP mutations diagrammed in Figure 1 and listed in Table II were expressed transiently in RCS cells. The intracellular distribution of expressed COMP protein detected by immunostaining of the FLAG epitope tag was used as the measure for intracellular trafficking behavior. In the previous study, we established the delayed trafficking of D469del mutant COMP using quantitative immunoblot analysis and fluorescence microscopy-based screening. Here, we focus on characterizing the trafficking behavior using morphological, fluorescence-based screening, since it is the more sensitive approach and allows evaluation of chondrocytes at the level of the individual cell. As shown in Figures 2 and 3, COMP expressed from the WT plasmid localizes predominantly to the juxtanuclear Golgi complex, with some protein in the ER (Figs. 2A,B and 3A,C). COMP immunostaining in the Golgi complex was verified by co-distribution of the Golgi specific marker, TGN38 (Fig. 3A–C). In contrast, D473G COMP, expressed from a mutation causing PSACH, is retained in the ER and absent from the Golgi complex (Figs. 2C,D and 3D,F) in a manner indistinguishable from that of the most common PSACH mutation, D469del mutant COMP [Chen et al., 2004]. No COMP immunostaining was detected in the region of the Golgi complex, which is defined by the TGN38 signal (Fig. 3D–F). For the T585M mutation, which causes MED, expressed COMP exhibits a more heterogenous localization pattern. T585M COMP was localized to the Golgi complex and ER in some cells, while in other cells the mutant protein was absent from the Golgi region (Fig. 2E,F). Immunolocalization results suggest that the clinical severity of individual COMP mutations is related to the degree of defective intracellular trafficking.

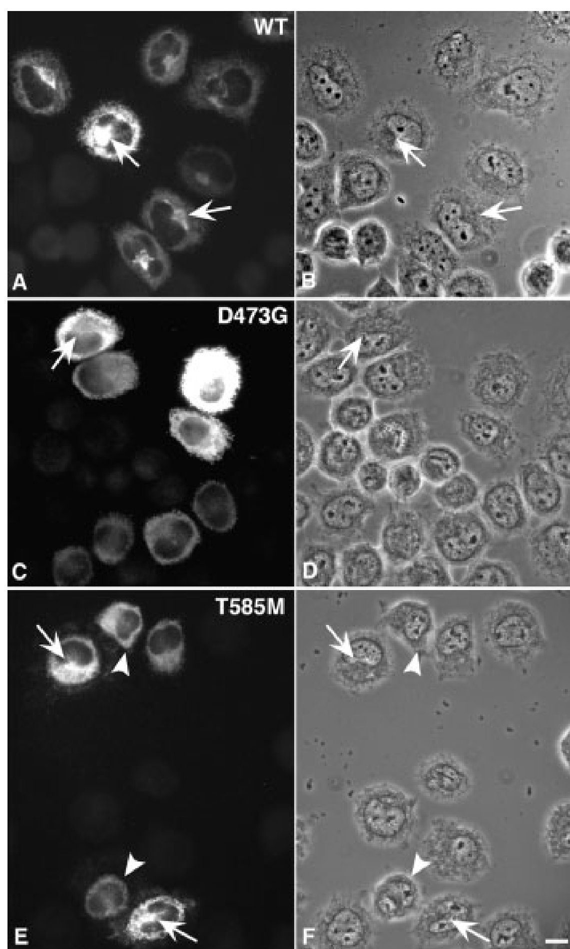

Fig. 2. RCS cells expressing WT or mutant COMP exhibit a variable degree of defective intracellular trafficking. Immunolocalization of expressed COMP was used as the tool to analyze the trafficking behavior of different mutant COMPs. WT COMP localizes to the juxtanuclear Golgi complex (arrows) of the RCS cells, with some protein in the ER (**A**). Independent verification that the juxtanuclear region corresponds to the Golgi complex is provided in Figure 3. D473G, a COMP mutation that causes PSACH, is observed only in the ER and is absent from the Golgi complex (**C**). For the COMP mutation T585M, which causes MED, expressed proteins are localized to the Golgi complex (arrows) and ER in some cells, while in other cells (arrowheads) very little immunofluorescence signal is detected in the Golgi region (**E**). In all cases, arrows denote the region of the juxtanuclear Golgi complex. Corresponding phase micrographs are shown in (**B**,**D**,**F**). Calibration bars in this figure and Figure 3 represent 10 μm.

We evaluated the intracellular localization of expressed COMP quantitatively to further

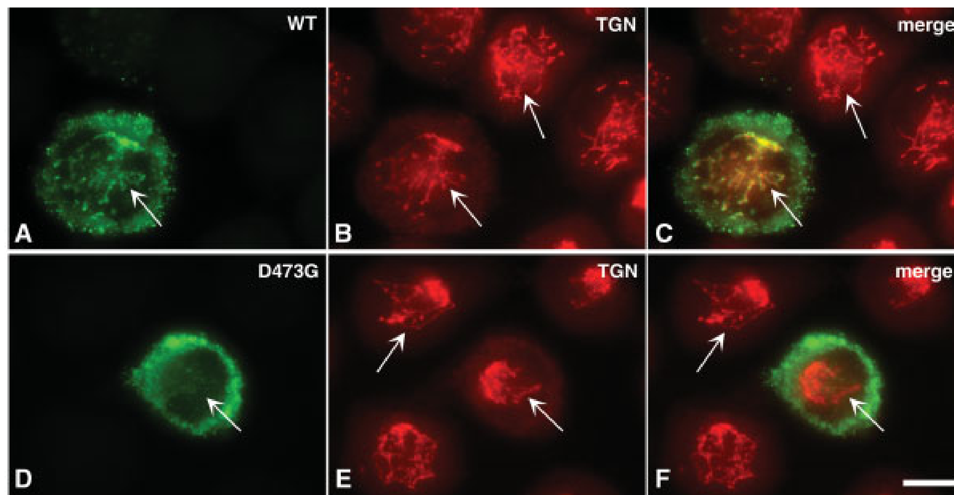

Fig. 3. WT COMP is co-distributed in the juxtanuclear Golgi complex with the Golgi-specific marker, TGN38, but mutated D473G COMP (PSACH) is not. Identity of the Golgi complex in the juxtanuclear region (arrows) is established by immunolocalization of the Golgi-specific marker, TGN38 (**B**,**E**). WT COMP is immunolocalized to both the ER and Golgi complex of expressing cells (**A**) while D473G COMP is present only in the ER and absent from the juxtanuclear region occupied by the Golgi complex (**D**). The merged images highlight the co-localization of WT COMP (**C**) and lack of co-localization of D473G COMP with TGN 38.

characterize the relationship between defective trafficking of expressed mutant COMPs in RCS cells and the clinical severity of COMP mutations. For each COMP mutation, and the WT control, cells were scored according to the presence or absence of expressed COMP immunostaining in the Golgi complex. Based on the percent of expressing cells characterized by significant Golgi immunolocalization, three major groups of COMP mutants were distinguished from the WT COMP, which localized to the Golgi complex in more than 95% of the expressing cells (Table III). Group 1, which includes D302V and P276R mutants, exhibited a mild defect in intracellular trafficking; expressed COMP localized to the Golgi complex in more than 85% of the expressing cells. Group 2 mutants, G299R, L272P, G309R, T585M, T585R, and H587R, exhibited an intermediate or moderate defect of intracellular trafficking; expressed COMP was localized to the Golgi complex in 20–60% of the scored RCS cells. The

TABLE III. Percentage of RCS Cells Expressing Wild Type or Mutant COMP Immunolocalized in the Golgi Complex

Mutations	COMP in Golgi	N	Clinical phenotype	Domain
WT	97.3 ± 3.6%	435		
Group 1				
D302V	91.7 ± 10.7%	566	MED	T3
P276R	85.7 ± 6.3%	385	MED	Linker T2–T3
Group 2				
G299R	48.8 ± 13.2%	481	PSACH	T3
L272P	47.3 ± 14.1%	464	PSACH	Linker T2–T3
G309R	35.8 ± 20.9%	417	PSACH	T3
H587R	63.4 ± 17.6%	567	PSACH	CTD
T585M	52.4 ± 14.1%	441	MED	CTD
T585R	23.1 ± 10.4%	409	MED	CTD
Group 3				
D271H	6.7 ± 3.0%	566	PSACH	Linker T2–T3
D469del	5.6 ± 7.7%	486	PSACH	T3
D473G	3.4 ± 3.0%	442	PSACH	T3
D473N	3.2 ± 2.6%	325	PSACH	T3

RCS cells expressing WT or mutant COMP were scored and categorized according to the presence of COMP immunostaining in the Golgi complex. The total number of expressing cells scored for each mutant is listed in the column under “N”. Data for the three CTD mutations are presented at the end of Group 2, as indicated.

third group, D469del, D473G, D473N, and D271H, exhibited severe defects in intracellular trafficking; expressed COMP was absent from the Golgi complex in more than 90% of expressing cells. All three CTD mutants, T585R, T585M, and H587R, exhibited an intermediate trafficking defect and fell into Group 2. In view of the significant structural differences between the T3 repeat/linker calcium binding regions and the CTD [Kvansakul et al., 2004], data for the COMP mutations were plotted separately according to the respective domain (Fig. 4A,B). When the T3/linker mutation data were examined, the relationship between the degree of the trafficking defect and severity of the clinical phenotype was evident (Fig. 4A). In contrast, no obvious association was noted between the trafficking behavior of mutated COMP and the clinical phenotype for mutations in the CTD (Fig. 4B).

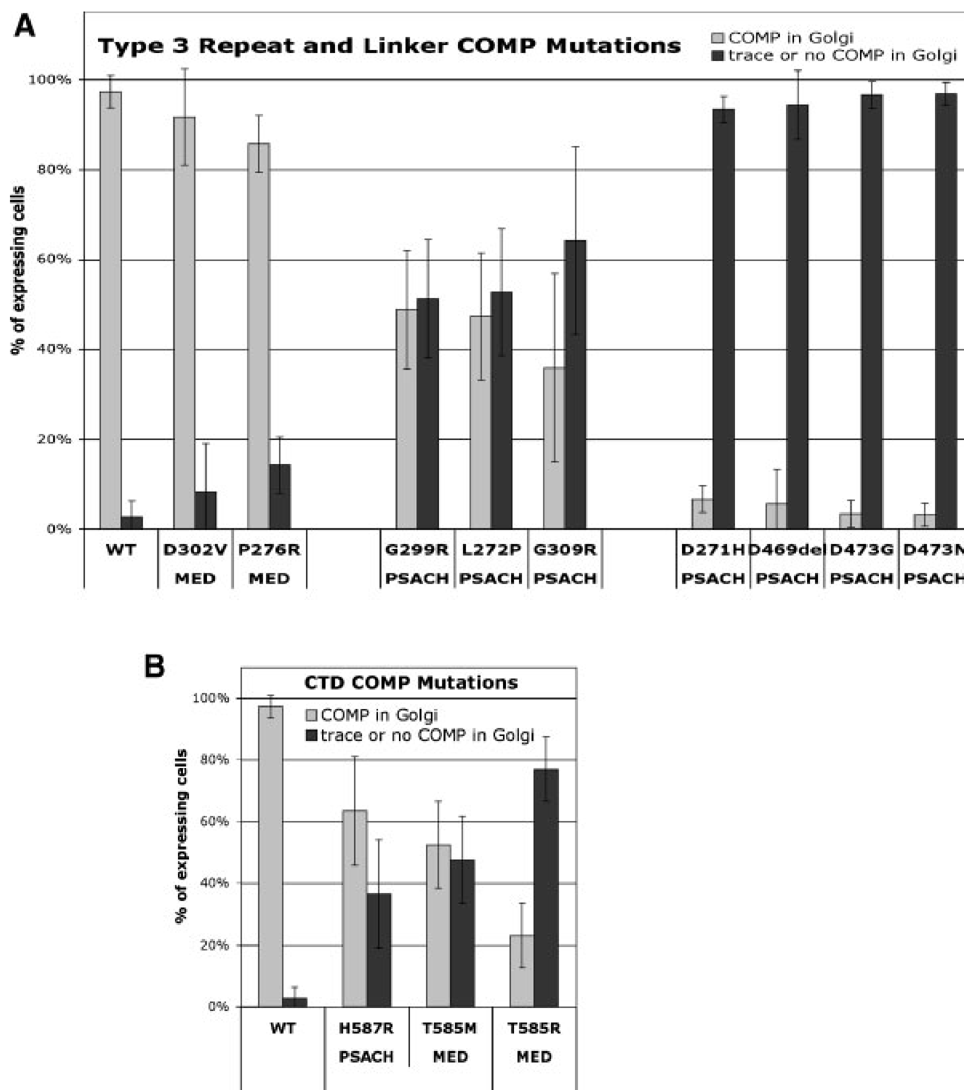

Fig. 4. The trafficking defect exhibited by COMP with T3 mutations is associated with severity of the corresponding clinical phenotype. Trafficking behavior of COMP mutations expressed in RCS cells is depicted by the percent of expressing cells with or without immunolocalized COMP in the Golgi complex and sorted according to the percent of expressing cells characterized by significant Golgi immunolocalization. **A**: For COMP mutations in T3 repeats and the linker region calcium binding domain, the extent of defective intracellular trafficking is closely associated with the corresponding clinical phenotypes. Individual mutations are presented according to the groups distinguished in Table II: Group 1, MED, least severe (D302V, P276R); Group 2, intermediate severity (G299R, L272P, G309R); and Group 3, PSACH, most severe (D271H, D469del, D473G, D473N). **B**: For COMP mutations in the CTD (H587R, T585M, T585R), however, the cellular trafficking defects led to designation in Group 2, intermediate severity, and appeared to be unrelated to the clinical phenotypes.

DISCUSSION

In this study, we evaluated the effect of COMP mutations in the T3 and CTD domains on the trafficking of COMP through the RCS chondrocytic secretory pathway. We tested 12 COMP mutations that presented within the range of mild (MED) to severe (PSACH) clinical symptoms, including the D469del mutation that was the subject of our previous communication [Chen et al., 2004]. Interestingly, when mutations were in calcium-binding (mostly T3 repeat) domains, a clear relationship between the in vitro cellular trafficking defect and clinical severity was observed. WT COMP was localized to the Golgi complex in 95% of RCS cells. The clinically less severe MED mutations reduced the percent RCS cells with Golgi-localized COMP to 85–90%, whereas PSACH-mutated protein was observed in the Golgi complex for only 5–50% of the cells. In striking contrast, no corresponding relationship to clinical severity was found for COMP with mutations in the CTD. Differences in the functional properties of the T3 and CTD domains may partially explain the variations observed in trafficking.

COMP domains have been defined byhomology, and to a lesser degree, function (Fig. 1). At the N-terminus is a coiled-coil domain responsible for forming the COMP homopentamer through intermolecular disulfide bonds. Four EGF-like T2 repeats, a linker region and seven T3 repeats encoding 13 calcium-binding motifs (DXDXDXXXDXXD) follow, with a globular domain at the C-terminus (CTD) [Oldberg et al., 1992; Newton et al., 1994; Adams et al., 1995].

The calcium-binding T3 domains are important structural elements that provide molecular stability [Kvansakul et al., 2004; Carlson et al., 2005]. These regions are highly conserved in all members of the thrombospondin family and contain over 85% of the COMP mutations [Adams et al., 1995]. The X-ray crystal structure of the C-terminal region of TSP-1, which includes $T3_{5-7}$ and the CTD, details how calcium ions form a central core that stablizes the protein [Kvansakul et al., 2004]. When COMP mutations are superimposed on the TSP-1 structure, most of the disease-causing mutations map near positions of calcium interactions. In turn, the perturbation of calcium coordination is predicted to disrupt protein folding.

In this study, we observed a consistent relationship between the degree of defective cellular trafficking in expressing cells and the severity of the clinical phenotype for mutations in the calcium-binding domains of the linker and T3 repeats. D271H, D469del, D473G, and D473N mutations, which all cause PSACH, exhibited the most profound retardation of COMP cellular trafficking. The PSACH mutations L272P, G299R, and G309R exhibited an intermediate level of delayed trafficking, whereas the milder MED disease mutations D302V and P276R delayed COMP transport from the ER to the Golgi complex only slightly more than the WT control (Fig. 4). Although all of these mutations are in or surrounding the EF-hand calcium binding motifs, some mutations affect the calcium binding coordinates while others do not [Adams et al., 1995]. Using the TSP-2 crystal structure and calcium co-ordination as a model, the effect of mutations is most likely related to the type and position of amino acid substitution and the number of calcium co-ordinations (e.g., one or two calcium ions, water mediation or main chain interaction) contributed by the residue [Carlson et al., 2005]. It is interesting that PSACH mutations D271H, D469del, D473G, and D473N, which are predicted to be in X-co-ordinate amino acids and located at positions that make bidentate connections to two calcium ions, exhibit severe trafficking defects, while L272P, G299R, and G309R mutations, which are in spacer amino acids between the co-ordinates and do not directly interact with calcium ions, cause less adverse trafficking defects. The two MED mutations minimally impair trafficking, but D302V is a Y-coordinate mutation and P276R is in a spacer region of the EF-hand. These considerations suggest that severity is also related to the neighborhood of the mutation and to factors other than the exact position in the calcium-binding motif.

In contrast to the T3 domain, the cellular trafficking behavior of CTD-mutated COMP in our experimental model, although reproducible, was not predictive of clinical severity. Most surprisingly, T585R-mutated COMP, which causes MED, exhibited an extreme cellular trafficking defect, while the T585M mutation, also classified as MED, led to an intermediate degree of delayed trafficking (Fig. 2). Interestingly, when threonine 585 is replaced by methionine, another hydrophobic amino acid, trafficking

is much less perturbed than by substitution with arginine, which is positively charged and consequently less conservative. The H587R PSACH mutation resulted in a milder than expected level of delayed trafficking, as was also reported by Schmitz et al. [2006] using a bovine chondrocyte model. In this case, the substitution does not change the polarity or charge on the amino acid, suggesting that this mutation may have a minimal effect [Berg et al., 2006]. The unpredictable behavior of CTD mutations in our experimental model and in the bovine model may reflect our limited understanding of the relevant structural and functional features of the CTD and the consequences of specific mutations. Perhaps mutations in the CTD, like those in the T3 repeats, can impose serious structural instabilities that interfere with protein folding. This idea is consistent with the compact assembly model of the CTD and last three T3 repeats [Kvansakul et al., 2004] and compatible with the suggestion that mutations in a CTD cluster at residues R718 and G719 (not included in our study) may cause destabilization of the T3-CTD interface [Kennedy et al., 2005b]. For those COMP mutations that only partially impair secretion, clinical severity is likely to be the consequence of a perturbation in the (abnormally) assembled cartilage extracellular matrix as well.

In summary, we have used our RCS in vitro cell model to assess the disease severity of COMP mutations. Our results indicate that mutations in the T3 repeat domain, regardless of the particular calcium-binding pocket, correlate with the MED/PSACH disease spectrum, while mutations in the CTD do not. These findings confirm and extend previous cellular mutation studies [Chen et al., 2004; Schmitz et al., 2006] and emphasize the need for further studies of the CTD domain and for an understanding of both intracellular and extracellular effects of mutated COMP proteins. Although the RCS model system provides an excellent assessment of cellular trafficking, an evaluation of extracellular matrix abnormalities will require a model that accurately reproduces the cartilage extracellular matrix as demonstrated by cultured PSACH patient chondrocytes or by overexpression in human chondrocytes [Hecht et al., 2005; Merritt et al., 2006, 2007].

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