SECRETION OF CARTILAGE OLIGOMERIC MATRIX PROTEIN IS AFFECTED BY THE SIGNAL PEPTIDE

Paul Holden¹,², Douglas R Keene¹,², Gregory P Lunstrum¹, Hans Peter Bächinger¹,³ and William A Horton¹,²

¹Research Center, Shriners Hospitals for Children, Portland, OR 97239, USA. ²Department of Molecular and Medical Genetics, Oregon Health & Science University, Portland, OR 97239, USA. ³Department of Biochemistry and Molecular Biology, Oregon Health & Science University, 3101 SW Sam Jackson Park Road, Portland, OR 97239, USA

Running title: The COMP Signal Peptide and Secretion

Address correspondence to: William A. Horton, Research Center, Shriners Hospitals for Children, Portland, OR 97239, USA, Tel. 503 221-1537; Fax. 503 221-3451; Email: wah@shcc.org

Cartilage oligomeric matrix protein (COMP) is a secreted glycoprotein found in the extracellular matrices of skeletal tissues. Mutations associated with two human skeletal dysplasias, pseudoachondroplasia (PSACH) and multiple epiphyseal dysplasia (MED) disturb COMP secretion leading to intracellular accumulation of mutant COMP, especially in chondrocytes. We show here that the manifestation of this secretory defect is dramatically influenced by the signal peptide that targets COMP for secretion. Comparison of wild type and mutant COMP secretion directed by the COMP or BM40 signal peptide in HEK-293 cells and rat chondrosarcoma cells revealed that the BM40 signal peptide substantially enhances secretion of mutant COMP that accumulates in endoplasmic reticulum (ER)-like structures when targeted by its own signal peptide. Additionally we demonstrate that mutant COMP forms mixed pentamers with wild type COMP. Our findings suggest that the secretory defect in PSACH and MED is not specific for chondrocytes, nor does it require interaction of mutant COMP with other matrix proteins prior to transport from the cell. They also imply a previously unappreciated role for the signal peptide in the regulation of protein secretion beyond targeting to the ER.

Cartilage oligomeric matrix protein (COMP) is a large pentameric glycoprotein found in the extracellular matrix of cartilage, tendon, ligament, synovium and in the vasculature (1-5). It is the fifth member of the thrombospondin family of proteins and as such has a modular domain structure comprised of an amino terminal domain, four EGF-like domains, eight-thrombospondin type III repeats and a C-terminal globular domain (6). While the complete biological role of COMP remains to be elucidated several specific domain functions have been identified. The amino terminal domain is the site of pentamerisation forming an alpha-helical coiled coil structure stabilized by disulphide bonds (2,7). Vitamin D₃ and retinoic acid have been shown to bind within the central hydrophobic pore of this structure in vitro suggesting a role for COMP as a storage and delivery protein for these molecules that are important in bone development and metabolism (8,9). Little is known of the function of the EGF-like repeats although it is thought that they are involved in calcium binding as is the case for the type III repeats. The globular C-terminal domain has been shown to interact with the fibrillar collagen types I and II and the FACIT collagen type IX in the presence of divalent cations (10-12). These findings suggest a role for COMP in the assembly of the extracellular matrix, an implication that is bolstered by the fact that COMP synthesis appears to be up-regulated by joint movement in the developing embryo and upon joint loading in tendon and also that proteolytic fragments of COMP are released into joint synovial fluid upon joint degeneration (13,14).

The biological importance of COMP was first realized by the genetic linkage of two human skeletal dysplasias, pseudoachondroplasia (PSACH) and multiple epiphyseal dysplasia (MED) to the COMP gene locus (15,16). Both
conditions are characterized by disproportionate short stature, ligamentous laxity and early onset osteoarthritis. Most disease causing mutations are found within the type III repeat region and result in single amino acid substitutions or deletions (17-19). Mutations have recently been detected in the C-terminal domain (20,21).

Several consequences of COMP mutations have been identified. Ultrastructural analysis of patient tissues has revealed massive accumulation of COMP within distended rough endoplasmic reticulum (rER) of chondrocytes and to a lesser extent in other COMP producing cells (19,22-24). Other matrix proteins known to interact with COMP, including collagen types IX, XI and XII, fibromodulin and decorin have been co-localized with COMP to enlarged chondrocyte rER in patient tissues as well (25). These observations have led to speculation that extracellular deficiency of COMP and potentially of COMP-interacting proteins is responsible for the cartilage disease associated with COMP mutations. There is also evidence that chondrocytes undergo premature death by apoptosis in PSACH, presumably because of the adverse effects of the intracellular accumulations (26,27). Although much controversy remains over which of these potential mechanisms is most relevant to the molecular pathogenesis of PSACH and MED, there is little doubt that they reflect an underlying disturbance of COMP secretion in cells that synthesize COMP, especially chondrocytes.

Investigation of defective COMP secretion in chondrocytes has been hampered by the limited availability of relevant patient and control human tissues, the lack of an informative animal model for PSACH or MED and the tendency of chondrocytes to dedifferentiate in culture. Most efforts to delineate the disturbance have involved expression of COMP as a recombinant protein in cultured cells. In many instances the BM40 signal peptide derived from osteonectin has been used in place of the native COMP signal peptide to direct nascent COMP chains into the secretory pathway (12,27,28). This expression strategy has gained popularity and is widely used because of its high yield of recombinant protein (29,30). However, we have uncovered evidence that this substitution of the signal peptide may mask the secretory disturbance caused by PSACH and MED mutations.

The signal peptide, a short stretch of approximately 12-30 amino acids, is found at the amino terminus of all proteins destined for secretion from the cell (31). Its primary function has long been considered to be to target proteins to the ER and subsequently into the secretory pathway. This targeting results from a cascade of molecular interactions initiated by the emergence of the signal peptide from the translocating ribosome. Summarily the signal peptide is recognized and bound by the cytosolic signal recognition particle which in turn binds its receptor at the ER membrane thus bringing the translating ribosome into contact with the translocon and facilitating insertion of the nascent chain into the translocon channel for passage through the lipid bilayer and into the lumen of the ER (32). While signal peptides all carry out this function analysis of their primary structures reveals little or no sequence homology and a remarkable diversity with regards to length, hydrophobicity and net charge (33). However, a common feature of signal peptides is a tripartite domain structure composed of a highly diverse N-terminal region, a central region of 7-15 hydrophobic residues and a short stretch of 2-9 small polar residues wherein lies the motif for cleavage by signal peptidase (34).

Since the initial discovery of the primary function of the signal peptide, several independent studies have uncovered further complexities of the translocation process and roles for the signal peptide (35,36). For example sequences in the protein domain being translocated can alter the efficiency of translocation by affecting various signal-receptor interactions. Signal peptides also appear to control gating of the protein translocation channel in a substrate specific manner and may carry out additional biological functions following their liberation from the nascent chain and cleavage by signal peptide peptidase (37,38). More recently it has been demonstrated that signal peptides initiate the pathway of maturation of the nascent chain in the ER including the addition of N-linked glycans that help to modulate oligomerisation, quality control and secretion of protein (39,40).

In this report we compare BM40 signal peptide to native COMP signal peptide directed secretion of wild type (WT) COMP and two mutant forms of COMP primarily in HEK-293
cells. The mutations del D469 and del RG367-368 represent PSACH and MED respectively (12,41). Our investigation was prompted by noting a discrepancy in secretion of recombinant COMP harboring the del D469 mutation between our observations and those reported by Dinser et al (12,27). They detected a relatively mild secretory defect using the BM40 signal peptide while we observed a severe defect utilizing the native COMP signal peptide. Our subsequent results document a substantial influence of the signal peptide on the secretion of mutant COMP. They suggest that use of the BM40 signal peptide directs nascent COMP chains into an alternative secretory pathway that may bypass one or more checkpoints that detect mutations that cause PSACH and MED.

Materials and Methods

Construction of wild type and mutant COMP - RNA was isolated from chondrocytes derived from the transgenic Col2-GFP reporter mouse strain (42). First strand cDNA was then synthesized from this RNA according to standard protocols using the Superscript system (GIBCO). The cDNA coding for mouse COMP was then amplified in 3 segments using the following primers: (1) 1-840 KpnI F (5'-TGGGTACCGCCATGGGCCCCACTGCC-3'), (2) 1-840 HindIII R (5'-GCAGCGAAGCTTCTCGTC-3'), (3) 823-1475 HindIII F (5'-GACGAGAAGCTTCGCTGC-3'), (4) 823-1475 MscI R (5'-TCCTCTTGGCCAGGGTTA-3'), (5) 1458-2268 MscI F (5'-TACCCCTGCGCCAAGAGGA-3') and (6) 1458-2268 XbaI R (5'-TCTAGAGACTCTCTGACGCCGTTG-3'). The PCR products were cloned into pCR2.1 and correct sequences confirmed by sequencing (ABI Prism 3100-Avant; Applied Biosystems). The expression vector pcDNA™V6His-V5 was digested with KpnI and XbaI and each fragment of COMP was then amplified in 3 segments using the following primers: (1) 1-840 KpnI F (5'-TGGGTACCGCCATGGGCCCCACTGCC-3'), (2) 1-840 HindIII R (5'-GCAGCGAAGCTTCTCGTC-3'), (3) 823-1475 HindIII F (5'-GACGAGAAGCTTCGCTGC-3'), (4) 823-1475 MscI R (5'-TCCTCTTGGCCAGGGTTA-3'), (5) 1458-2268 MscI F (5'-TACCCCTGCGCCAAGAGGA-3') and (6) 1458-2268 XbaI R (5'-TCTAGAGACTCTCTGACGCCGTTG-3'). The PCR products were cloned into pCR2.1 and correct sequences confirmed by sequencing (ABI Prism 3100-Avant; Applied Biosystems). The expression vector pcDNA™V6His-V5 was digested with KpnI and XbaI and each fragment of COMP was then amplified in 3 segments using the appropriate enzymes as noted in the primer name and ligated into the digested pcDNA™V6His-V5. Del D mutation cDNA was generated by PCR using primer 3 and mutagenesis primer del D R (5'-GTCATCATCGTCACAGGCATCGCC-3'). A separate reaction was performed with mutagenesis primer del D F (5'-GGCGATGCCTGTGACGATGAC-3') and primer 4. These PCR products were then used as a template for a final PCR with primers 3 and 4 to amplify del D cDNA. Del RG mutation cDNA was generated by PCR using primer 3 and mutagenesis primer del RG R (5'-GTCGAGGCCATCGCCATCCAGGTC-3'). A separate reaction was performed with mutagenesis primer del RG F (5'-GACCTGGATGCGTGCAGTCGAC-3') and primer 4. These PCR products were then used as a template for a final PCR with primers 3 and 4 to amplify del RG cDNA.

To replace the COMP signal peptide with that of BM40 the 5’ end of COMP was re-amplified with primer BM40 1 (5'-CCCCGCTAGCCAGGCGCCAGATCC-3') incorporating an NheI site at the 5’ end and primer (2) resulting in the generation of the 5’ end of COMP lacking the signal peptide coding region. The PCR product was digested with NheI and XhoI and cloned into the expression vector pCEPγ/II that contains the sequence for the BM40/SPARC signal peptide (30) thus generating a plasmid containing the 5’ end of COMP bearing the BM40 signal peptide. This was then digested with KpnI and XhoI to excise the BM40 signal-COMP fragment. Existing COMP constructs were also digested with KpnI and XhoI to excise the 5’ end of COMP. The BM40 signal-COMP fragment was then ligated into these linearised vectors generating wild type and mutant COMP constructs bearing the BM40 signal peptide.

To replace the C-terminal 6 histidine tag with a FLAG tag the following primers were used: BAM 1 (5'-GGAAGGATCCTCGAAACG-3') containing a 5’ BamHI site and FLAG R AgeI (5'-CCGGTTCACTTATCGTGCATCCTTTGAATTTCGCGTGAATCGAGACCGAGGAG-3') incorporating a 3’ AgeI site. These primers were used in a PCR reaction with WT-COMP pcDNA™V6His-V5 as a template generating a fragment comprising the 3’ end of COMP and the 3’ end of the MCS of pcDNA™V6His-V5 with a FLAG tag followed by a stop codon and an AgeI site 5’ of the His-tag. Following sub-cloning into pCR2.1 and sequence confirmation this fragment was then cloned into each of the pre-existing COMP constructs that had been linearised by digestion with BamHI and AgeI.
Expression and analysis of recombinant protein -

The pcDNA®6/V5-His vector (Invitrogen) harboring wild type or mutant COMP cDNA with the COMP or BM40 signal peptide and a C-terminal His-tag or FLAG tag was transfected into HEK-293 cells using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s protocol. For transfection of rat chondrosarcoma (RCS) cells the cells were seeded at a density of 4 x 10^5 cells per well of a 6 well plate and cultured for 4 hours in culture medium containing 4 units/ml of bovine testicular hyaluronidase (Sigma). This medium was removed and Lipofectamine™ 2000 was then used for transfection according to the manufacturer’s protocol but in the presence of 4 units/ml of hyaluronidase. For establishment of stably transfected cell lines transfected cells were harvested by trypsinisation 48 hours post transfection and re-seeded at a dilution of 1:10 into fresh medium containing blasticidin S-HCl (Invitrogen) at a concentration of 10 µg/ml. For expression analysis of transiently transfected cells, culture medium was harvested 48 hours post transfection and cell lysates were prepared in ice cold lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl) containing protease inhibitors (1 complete, mini, EDTA-free tablet (Roche) per 7 ml of lysis buffer). Cells were harvested by trypsinisation and then lysed by vortexing for 15 seconds in lysis buffer followed by incubation on ice for 20 minutes and centrifugation for 10 minutes at 16,000 xg at 4°C. Harvested culture medium was cleared of detached cells and debris by centrifugation for 2 minutes at 1,500 xg. Proteins were analyzed in the presence (reduced) or absence (non-reduced) of 100 mM dithiothreitol by SDS-PAGE using 8% Novex® Tris-Glycine gels, 4-12% Novex® Tris-Glycine gels (Invitrogen) or 3-8% Novex® Tris-Acetate gels. Following electrophoresis proteins were transferred from gels to nitrocellulose by standard protocol. In most cases the nitrocellulose was probed with HRP-conjugated anti-His mouse monoclonal antibody (Invitrogen) at a dilution of 1:5000 or HRP-conjugated anti-FLAG mouse monoclonal antibody (Sigma) at a dilution of 1:5000 or anti-GAPDH mouse monoclonal antibody (Novus Biologicals) at a dilution of 1:1000. Detection was then performed using the enhanced chemiluminescence western blotting analysis system (Amersham) according to the manufacturer’s protocol. In cases where quantification was required the enhanced chemifluorescence system (Amersham) was used. In these cases the nitrocellulose was probed with anti-His mouse monoclonal antibody (Invitrogen) at a dilution of 1:5000 followed by anti-mouse IgG fluorescein linked whole antibody (Amersham) at a dilution of 1:600 and finally with anti-fluorescein alkaline phosphatase conjugate (Amersham) at a dilution of 1:2500. Signal was then generated by addition of ECF substrate to the membrane. For both ECL and ECF detection, membranes were read using the STORM system (Molecular Dynamics) and for quantification ECF generated bands were analyzed using Image Quant analysis software. In pulse-chase experiments samples were electrophoresed on 8% Tris-Glycine gels under reducing conditions. The gels were then fixed in 50% methanol and 10% acetic acid for 1/2 hour and washed extensively in water prior to drying onto filter paper using a vacuum gel dryer (Biorad). Dried gels were exposed overnight to a low energy screen (Kodak) prior to image analysis. The predominant bands present in the culture media gels were identified as COMP by western blot. The COMP bands in the lysate gels were identified by aligning them with the bands in the culture media gels.

Confocal microscopy - Transiently transfected HEK-293 cells were plated onto 18 mm glass cover slips in 12 well culture plates 48 hours post-transfection. For co-localization experiments HEK-293 cells stably expressing the ER targeting sequence of calreticulin fused to the 5’ end of Discosoma sp. red fluorescent protein (DsRed2) and the ER retention sequence, KDEL (pDsRED2-ER, Clontech) were used. Following transfection cells were allowed to adhere to the cover slips overnight and then were rinsed with PBS prior to fixation in 4% paraformaldehyde in PBS for 15 minutes at 4 ºC. Fixed cells were washed three times with PBS and incubated in blocking buffer (PBS, 0.1% saponin, 5% heat denatured goat serum) for 2 hours at room temperature. Cells were then incubated with anti-His mouse monoclonal antibody (Invitrogen) at a dilution of 1:400 for one hour at room temperature in PBS containing 0.1% saponin and 2% heat denatured goat serum. Cells were washed with 3 changes of
PBS containing 0.1% saponin and then incubated with Alexa fluoro 488 anti mouse IgG (H+L) secondary antibody (Molecular Probes) at a dilution of 1:200 in PBS containing 0.1% saponin and 2% heat denatured goat serum in the dark for one hour at room temperature. Cells were then washed with 3 changes of PBS containing 0.1% saponin and finally rinsed with distilled H_{2}O. Cover slips were mounted onto glass slides using vectashield (Vector) and viewed using a Leica TCS SP2 confocal microscope system (Leica).

**Transmission Electron Microscopy** - Cell cultures that were duplicates of those processed for confocal microscopy were pelleted and rinsed in Dulbecco’s serum-free media and fixed in media-buffered 1.5% glutaraldehyde/1.5% paraformaldehyde for 60 minutes at 4°C. Following another rinse, the pellets were post-fixed in media-buffered 1% OsO_{4} for 60 minutes at 4°C, rinsed in media, dehydrated in a graded alcohol series to 100%, rinsed in propylene oxide and embedded in Spurr’s epoxy. Sections (70 nm thick) were mounted on formvar-coated grids and photographed using a Philips 410LS TEM operated at 80 KV.

**Pulse-Chase analysis** - HEK-293 cells stably transfected with COMP constructs were seeded into the wells of 6 well plates (3 x 10^6 cells per well) and cultured overnight. Culture medium was removed and the cells washed twice with PBS. Serum free culture medium lacking cysteine and methionine (Sigma) was added and the cells cultured for 2 hours. This medium was then removed and replaced with serum free medium containing 50 μCi per ml of S^{35} methionine and cysteine (MP Biomedicals, Inc.) and the cells were labeled for 1/2 hour (Pulse time). This medium was then removed, the cells were washed with PBS and serum free complete medium was then added. At set time points culture medium was removed and cell lysates were prepared as described earlier (Chase times).

**Purification of His-tagged protein** - His-tagged proteins were purified from culture medium and cell lysates using HIS-Select HF Nickel Affinity Gel (Sigma) according to the manufacturer’s protocol. Briefly for each sample 100 μl of affinity gel slurry was washed with 1 ml of water and then equilibrated in 1 ml of equilibration buffer (50 mM sodium phosphate pH 8.0, 0.3 M sodium chloride, 10 mM Imidazole). Culture medium was cleared by centrifugation for 2 min. at 1,500 xg and the pH adjusted by addition of 1/100 volume of 200 mM Tris-HCl pH 8.0. Equilibrated affinity gel was then added and incubated for 1 hour at 4°C with inversion. The gel was then recovered by centrifugation and washed in 3x 1 ml of equilibration buffer. Bound proteins were then eluted by addition of 100 μl of elution buffer (50 mM sodium phosphate pH 8.0, 0.3 M sodium chloride, 500 mM Imidazole).

**RESULTS**

The signal peptide affects secretion of wild type and mutant COMP. The PSACH-causing mutation del D469 and the MED-causing mutation del RG367-368 were introduced into the full-length mouse COMP cDNA by PCR-based mutagenesis. For simplicity throughout the remainder of this manuscript these mutations shall be referred to as del D and del RG respectively. The domain structure of a COMP monomer together with the approximate locations of each of the mutations is illustrated in figure 1A. Duplicate constructs were made wherein the native COMP signal peptide (residues 1-19) was replaced with that of BM40. This signal peptide was chosen since it has been used in several published studies assessing the secretion of mutant COMP. Since the hydrophobic region of the signal peptide has been shown to be important in the insertion of the signal peptide into the lipid bilayer of the rER and subsequent positioning of the cleavage site for access by signal peptidase, we compared the hydropathy profiles of the two signal peptides (43). As shown in figure 1B Kyte-Doolittle analysis of the COMP and BM40 signal peptides indicates that the hydrophobic portion of the COMP signal peptide is more hydrophobic than that of the BM40 signal peptide (44).

To determine the effect of the signal peptide on COMP secretion, each construct was transfected into HEK-293 cells that do not produce endogenous COMP. The relative abundance of COMP in cell lysates and culture media was assessed by western blot 48 hours later (Figure 2A). Wild type COMP was efficiently synthesized and secreted when the COMP signal peptide was employed as evidenced by a single band with an apparent molecular weight of 100 kDa in the
lysate and a doublet in the medium (Doublet caused by proteolysis within the amino terminal pentamerisation domain – P. Holden, unpublished results). In contrast mutant COMP was produced but not secreted at all (del D) or secreted at a much lower level than wild type COMP (del RG). In each case substitution with the BM40 signal peptide resulted in increased secretion of COMP as evidenced by a decrease in the intracellular amount of COMP and an increase in COMP in the medium. The experiment was repeated in triplicate and the amount of intracellular and extracellular COMP was quantified by densitometry (Figure 2B). Comparison of ratios of secreted to intracellular COMP revealed that the BM40 signal peptide markedly increased this ratio in each case as shown in figure 2B.

The intracellular distribution of COMP is affected by the signal peptide. Since the biochemical studies demonstrated that the BM40 signal peptide had a striking effect on COMP secretion we examined its effects on the intracellular location of COMP using confocal microscopy. Figure 3 shows that wild type COMP targeted to the secretory pathway by the signal peptide resided diffusely throughout the cell in small vesicular structures (Figure 3 – COMP signal peptide, WT). In contrast both del D and del RG COMP were located in large globular aggregates (Figure 3 – COMP signal peptide, del D and del RG). When the COMP signal peptide was replaced with the BM40 signal peptide no obvious difference was observed in the location of wild type COMP (Figure 3 – BM40 signal peptide, WT) but dramatic differences were noted for the mutant proteins. The large globular aggregates present when the mutant proteins were targeted by the COMP signal peptide were no longer evident rather the mutant COMP occupied small diffusely distributed vesicles much like those seen for wild type COMP (Figure 3 – BM40 signal peptide, del D and del RG). Similar results were observed for cells stably expressing the wild type and mutant COMP constructs (data not shown). To investigate whether or not the globular aggregates formed when mutant COMP was targeted by the COMP signal peptide represented ER we transiently transfected the constructs into HEK-293 cells that had previously been stably transfected with pDsRed2-ER (Figure 3 – Right panel). Wild type COMP predominantly colocalized with the ER marker although some did not displaying instead a juxtanuclear distribution possibly representing golgi. The globular aggregates formed by each mutant COMP also appeared to colocalize mainly with the ER marker. Mutant COMP accumulates in dilated rER-like structures. To confirm the ER localization of mutant COMP in this system, cultures that were duplicates of those viewed by confocal microscopy were analyzed by transmission electron microscopy (TEM). This revealed that cells expressing wild type COMP using the COMP signal peptide were unremarkable in terms of the appearance of the rER. In these cells the rER appeared mostly as thin ribosome studded ribbons and small studded vesicles (Figure 4 – WT). In contrast cells expressing del D and del RG COMP using the COMP signal peptide both contained what appeared to be dilated rER (Figure 4 – del D and del RG).

The BM40 signal peptide increases the efficiency of secretion of COMP. Since cells expressing wild type COMP targeted for secretion by the BM40 signal peptide had a higher ratio of extracellular to intracellular COMP than cells expressing wild type COMP targeted by the COMP signal peptide, we considered that the BM40 signal peptide might increase the efficiency of secretion of COMP. To investigate this possibility a pulse chase experiment was performed on cells stably expressing each construct and densitometry was used to quantify secreted COMP at each time point. We focused on the mutation del D as representative of previously published work. Figure 5A shows that when targeted by the BM40 signal peptide more wild type COMP is secreted at each time point than when wild type COMP is targeted by the COMP signal peptide. This difference was confirmed for secreted COMP by densitometric analysis of the bands (Figure 5B). Comparison of the secretion of wild type and del D COMP both targeted by the COMP signal peptide revealed that del D COMP was minimally secreted (This apparent discrepancy from the western blot analysis of culture medium from cells expressing del D COMP (Figure 2) where no COMP was detectable was attributed to the greater sensitivity of this technique). This relative lack of secretion was reflected in the cell lysates wherein del D COMP remained present up until and including the 24 hour chase time whereas wild
type COMP had mostly cleared from the cells by 2 hours (Figure 5A). In contrast secretion of del D COMP when targeted by the BM40 signal peptide was much greater, the protein being mostly cleared from the cells following 8 hours and the final secreted amount being approximately 5 times greater (Figure 5A and 5B). Furthermore comparison of the secretion of wild type COMP targeted by the COMP signal peptide and del D COMP targeted by the BM40 signal peptide revealed that the BM40 signal peptide brought the final level of secretion of del D COMP to roughly half that of wild type COMP at 24 hours (Figure 5B).

**Mutant COMP accumulates in HEK-293 cells.** To confirm that del D COMP continues to accumulate in HEK-293 cells replicate plates of stably transfected cells were set up in parallel with the pulse chase experiment and cultured at confluence for 6 days. The relative abundance of COMP in cell lysates and culture media was then assessed by western blot (Figure 6A). When del D COMP was targeted by the COMP signal peptide there was significant retention of protein and minimal secretion (Figure 6A). In accordance with our other experiments translocation of del D COMP by the BM40 signal peptide resulted in a substantial decrease in retention and a concomitant increase in secretion. While these effects were clearly apparent from the western blots densitometric quantification of triplicate samples revealed the full extent of the differences. Assigning a value of 1 to wild type protein targeted by the COMP signal peptide and adjusting values for the other constructs accordingly revealed that there was approximately 6 times as much intracellular del D COMP as wild type COMP and that the BM40 signal peptide reduced this amount by half (Figure 6B). Similarly there was approximately twice as much del D COMP secreted when targeted by the BM40 signal peptide as when targeted by the COMP signal peptide.

**Mutant COMP forms mixed hetero-pentamers with wild type COMP.** Since PSACH and MED are heterozygous conditions patients have one wild type allele and one mutant allele and thus presumably produce equal amounts of wild type and mutant COMP chains. While we have shown that mutant COMP secretion is retarded relative to wild type COMP secretion we next sought whether or not mutant COMP could form mixed hetero-pentamers with wild type COMP. Duplicate constructs were made wherein the C-terminal His-tags were replaced by FLAG tags. In all cases the COMP signal peptide was used. Various combinations of wild type His-tagged COMP and mutant FLAG-tagged COMP DNA in equal amounts were then transfected into HEK-293 cells. The relative abundance of COMP in cell lysates and culture media was assessed by western blot 24 hours later. Figure 7 shows that as seen earlier mutant COMP alone was barely secreted (del D) or its secretion was substantially reduced (del RG) relative to wild type COMP (Figure 7A). However when co-transfected with an equal amount of wild type COMP DNA the amount of mutant COMP secreted into the culture medium was increased in each case and the amount of wild type COMP secreted was reduced, reflecting retention of wild type COMP. To verify that the secreted mutant COMP was in a higher order assembly with wild type COMP, nickel affinity resin was used to purify the His-tagged wild type COMP. Analysis of the purified protein by western blot utilizing antibodies to the FLAG tag revealed the presence of FLAG tagged mutant COMP indicating that it was in a higher order assembly with wild type COMP since otherwise it would not have bound to the nickel resin (Figure 7B). To confirm the results of this experiment and also to exclude the possibility that the signal peptide effects that we have described were HEK-293 cell specific, we transiently transfected RCS chondrocytic cells with each construct (Figure 7C). In this case since RCS cells endogenously express COMP (2), the level of secretion of each mutant was higher than when the mutant protein was expressed alone in HEK-293 cells indicative of the formation of mixed heteropentamers, thus mirroring the results of the co-transfection experiment. Additionally use of the BM40 signal peptide in place of the COMP signal peptide resulted in greater secretion of mutant COMP and subsequently reduced retention (Figure 7C) thus confirming that the effect of the BM40 signal peptide was not specific to HEK-293 cells.
DISCUSSION

The improper folding of mutant proteins and their subsequent intracellular accumulation is the basis of many pathological conditions. PSACH and MED are two such conditions wherein COMP mutations have been shown to give rise to large intracellular accumulations of COMP together with other components of the cartilage extracellular matrix. In this report we have demonstrated that the disease causing mutations del D (PSACH) and del RG (MED) severely disrupt secretion of recombinant COMP in HEK-293 cells when the protein is directed into the secretory pathway by its own signal peptide. More strikingly we showed that substitution with the BM40 signal peptide largely ameliorated this disturbance.

We also documented that mutant COMP when translocated by its own signal peptide does accumulate in HEK-293 cells when cultured for prolonged periods of time. A recent study by Hashimoto et al also demonstrated a similar retention of mutant COMP (albeit a different mutation) when over-expressed in COS-7 cells (45). These data combined with our observations that the signal peptide had a similar effect on secretion of mutant COMP in chondrocytic RCS cells indicate that COMP retention is not a cell type specific phenomenon as was previously hypothesized.

A problem in the study of PSACH and MED patient tissue and cell samples is that there has been no way to distinguish between wild type and mutant COMP and thus the questions of whether or not mutant COMP is secreted at all and if it assembles in mixed pentamers with wild type COMP have remained unanswered. We have confirmed here that mutant COMP does form mixed pentamers with wild type COMP. This event could have two effects; firstly that wild type COMP would be retained intracellularly with mutant COMP. This in itself apparently would not drastically affect the extra-cellular matrix since the COMP null mouse displayed no apparent phenotype (46). Thus it is thought that the presence of COMP is not an absolute requirement for the development and maintenance of bones and cartilage, possibly due to the compensatory effect of another as-yet unidentified protein.

Secondly some of these mixed pentamers would be secreted resulting in the extracellular presence of mutant COMP that could conceivably interfere with the normal function of COMP. For instance a pentamer containing mutant chains may not be able to interact properly with other matrix components e.g. type IX collagen and this may lead to the formation of an improperly assembled cartilage matrix. Of particular interest is the potential effect of the MED causing del RG mutation (41). This mutation results in the deletion of the RG residues from the potentially integrin interacting RGD site in the third type III repeat of COMP. While the precise biological role of COMP remains unclear the possibility that it may interact with cells is suggested by the presence of this RGD site and a VVM motif within the C-terminal domain. These have been shown to mediate cell interactions in thrombospondin 1 (47). Thus extracellular COMP carrying the del RG mutation may not be able to interact with its potential receptor possibly leading to compromised stability of the cartilage matrix.

It is not clear how changing the signal peptide could affect the process of secretion so markedly. Our comparison of the hydropathy plots of each signal peptide revealed a difference in the location and size of the peak of hydropathy relative to the amino terminus of the signal peptide. The hydrophobic region of a signal peptide is thought to be important for targeting and efficient insertion of the signal peptide into the ER membrane (43,48). Recent papers have demonstrated that the nature of the initial association between the signal peptide and the translocon during the initiation of translocation also differs between signal peptides (36,37). This interaction is thought to be governed in part by information encoded in the hydrophobic portion of the signal peptide and has been shown to influence the timing and efficiency of maturational events, specifically signal peptide cleavage and glycosylation (39). However since so little is currently know about the precise function of signal peptides we cannot assume that the difference in hydropathy that we observed is the sole basis for the phenomenon described here. It is worth noting that the COMP and BM40 signal peptides also differ with respect to the position of their cleavage site relative to the amino terminus of the nascent chain. This could conceivably result in a
difference in the way that the cleavage site is presented to the lumenal signal peptidase that could subsequently affect the timing or efficiency of signal peptide cleavage. While in most cases signal peptide cleavage is thought to occur co-translationally, for some proteins it has been shown to occur inefficiently and very late after translocation across the ER membrane. For instance the HIV-1 gp160 envelope protein is a specific example of such a protein and also an example of the importance of a different region of the signal peptide. Basic amino acids in the N-terminal region of the HIV-1 gp160 signal peptide appear to be responsible for the slow cleavage that results in the retention of gp160 molecules bound to calnexin in the rER with their signal peptides still uncleaved (49).

Intracellular inclusions of mutant COMP found in PSACH chondrocytes also contain other matrix components, notably type IX collagen but interestingly no type II collagen. Type II collagen is an early marker of chondrogenesis and one of the first proteins to be secreted by differentiating chondrocytes. It has already been shown that type II collagen and aggrecan are processed along different secretory pathways (50). The fact that type II collagen is not co-retained with mutant COMP in PSACH chondrocytes implies that the two proteins do not encounter each other along the secretory pathway. We hypothesize that the signal peptide may direct its ‘passenger’ protein along a specific secretory pathway. Hydrophathy analysis of the type II collagen and aggrecan signal peptides revealed similar differences as between the COMP and BM40 signal peptides (not shown). These specific secretory pathways may differ in their complement of folding chaperones or in the nature of the folding environment which may explain the difference in quality control effects between mutant COMP translocated by the COMP signal peptide and mutant COMP translocated by the BM40 signal peptide. Additionally they may also serve as a mechanism to sort certain proteins that interact extracellularly thus facilitating the correct order of assembly.

On a broader scale recent work has demonstrated that signal peptides can also have post-targeting functions following their liberation from the nascent chain (38,51). As one example the cleaved signal peptide of bovine prepro-lactin is cleaved and then processed by an unknown signal peptide peptidase (52). This fragment is ultimately released into the cytosol where it then binds to calmodulin in a Ca$^{2+}$ dependent manner (53). Since calmodulin is involved in the regulation of many cellular processes via control of Ca$^{2+}$ dependent signaling pathways, it has been postulated that binding of specific signal peptide fragments to calmodulin may in some way influence such signaling pathways. While the physiological consequences of interactions between signal peptide fragments and calmodulin are not yet known there exists the potential for effects on many levels. The importance of calcium binding to the structure of COMP is well documented (54,55). In the case of COMP and other calcium binding proteins, cleavage of the signal peptide and subsequent interaction with calmodulin in the cytosol could conceivably stimulate an influx of Ca$^{2+}$ into the ER where it would then bind to COMP as it folds. The involvement of calcium in the unfolded protein response is also well documented (56). This response appears to fail in the case of mutant COMP resulting in accumulation rather than retrotranslocation into the cytosol for ubiquitination and degradation. The fact that the final level of quality control of COMP is so different when targeted by the BM40 signal peptide as opposed to the native COMP signal peptide implies an as yet unidentified role for signal peptides in protein quality control. That this may occur in some way at the level of a post cleavage function of the signal peptide represents an intriguing possibility.

In short there is a growing body of evidence that the degeneracy of a signal peptide is in fact a consequence of evolution with signal peptides being specifically optimized and tailored to guide the translocation, folding and secretion of their passenger protein. Our observations are consistent with this notion and illustrate that the signal peptide can be relevant to the molecular pathogenesis of a disease. Our findings also underscore the importance of using native signal peptides when investigating secretion of recombinant proteins in vitro.
REFERENCES

1. Hedbom, E., Antonsson, P., Hjerpe, A., Aeschlimann, D., Paulsson, M., Rosa-Pimentel, E., Sommarin, Y., Wendel, M., Oldberg, A., and Heinegard, D. (1992) J Biol Chem 267, 6132-6136
2. Morgelin, M., Heinegard, D., Engel, J., and Paulsson, M. (1992) J Biol Chem 267, 6137-6141
3. DiCesare, P., Hauser, N., Lehman, D., Pasumarti, S., and Paulsson, M. (1994) FEBS Lett 354, 237-240
4. Muller, G., Michel, A., and Altenburg, E. (1998) Connect Tissue Res 39, 233-244
5. Riessen, R., Fenchel, M., Chien, H., Axel, D. I., Karsch, K. R., and Lawler, J. (2001) Arterioscler Thromb Vasc Biol 21, 47-54
6. Oldberg, A., Antonsson, P., Lindblom, K., and Heinegard, D. (1992) J Biol Chem 267, 22346-22350
7. Malashkevich, V. N., Kammerer, R. A., Efimov, V. P., Schultthess, T., and Engel, J. (1996) Science 274, 761-765
8. Guo, Y., Bozic, D., Malashkevich, V. N., Kammerer, R. A., Schultthess, T., and Engel, J. (1998) Embo J 17, 5265-5272
9. Ozbek, S., Engel, J., and Stetefeld, J. (2002) Embo J 21, 5960-5968
10. Rosenberg, K., Olsson, H., Morgelin, M., and Heinegard, D. (1998) J Biol Chem 273, 20397-20403
11. Holden, P., Meadows, R. S., Chapman, K. L., Grant, M. E., Kadler, K. E., and Briggs, M. D. (2001) J Biol Chem 276, 6046-6055
12. Thur, J., Rosenberg, K., Nitsche, D. P., Pihlajamaa, T., Ala-Kokko, L., Heinegard, D., Paulsson, M., and Maurer, P. (2001) J Biol Chem 276, 6083-6092
13. Lohmander, L. S., Saxne, T., and Heinegard, D. K. (1994) Ann Rheum Dis 53, 8-13
14. Smith, R. K., Gerard, M., Dowling, B., Dart, A. J., Birch, H. L., and Goodship, A. E. (2002) Equine Vet J Suppl, 241-244
15. Briggs, M. D., Hoffman, S. M., King, L. M., Olsen, A. S., Mohrenweiser, H., Leroy, J. G., Mortier, G. R., Rimoin, D. L., Lachman, R. S., Gaines, E. S., and et al. (1995) Nat Genet 10, 330-336
16. Hecht, J. T., Nelson, L. D., Crowder, E., Wang, Y., Elder, F. F., Harrison, W. R., Francomano, C. A., Prange, C. K., Lennon, G. G., Deere, M., and et al. (1995) Nat Genet 10, 325-329
17. Briggs, M. D., and Chapman, K. L. (2002) Hum Mutat 19, 465-478
18. Unger, S., and Hecht, J. T. (2001) Am J Med Genet 106, 244-250
19. Maddox, B. K., Keene, D. R., Sakai, L. Y., Charbonneau, N. L., Morris, N. P., Ridgway, C. C., Boswell, B. A., Sussman, M. D., Horton, W. A., Bachinger, H. P., and Hecht, J. T. (1997) J Biol Chem 272, 30993-30997
20. Briggs, M. D., Mortier, G. R., Cole, W. G., King, L. M., Golik, S. S., Bonaventure, J., Nuytinck, L., De Paepe, A., Leroy, J. G., Biesecker, L., Lipson, M., Wilcox, W. R., Lachman, R. S., Rimoin, D. L., Knowlton, R. G., and Cohn, D. H. (1998) Am J Hum Genet 62, 311-319
21. Mabuchi, A., Manabe, N., Haga, N., Kitoh, H., Ikeda, T., Kawaji, H., Tamai, K., Hamada, J., Nakamura, S., Brunetti-Pierri, N., Kimizuka, M., Takatori, Y., Nakamura, K., Nishimura, G., Ohashi, H., and Ikegawa, S. (2003) Hum Genet 112, 84-90
22. Maddox, B. K., Mokashi, A., Keene, D. R., and Bachinger, H. P. (2000) J Biol Chem 275, 11412-11417
23. Hecht, J. T., Hayes, E., Snuggs, M., Decker, G., Montufar-Solis, D., Does, K., Mwalle, F., Poole, R., Stevens, J., and Duke, P. J. (2001) Matrix Biol 20, 251-262
24. Delot, E., Brodie, S. G., King, L. M., Wilcox, W. R., and Cohn, D. H. (1998) J Biol Chem 273, 26692-26697
25. Vranka, J., Mokashi, A., Keene, D. R., Tu, S., Corder, G., Sussman, M., Horton, W. A., Maddox, K., Sakai, L., and Bachinger, H. P. (2001) Matrix Biol 20, 439-450
26. Duke, J., Montufar-Solis, D., Underwood, S., Lalani, Z., and Hecht, J. T. (2003) *Apoptosis* **8**, 191-197
27. Dinser, R., Zaucke, F., Kreppel, F., Hultenby, K., Kochanek, S., Paulsson, M., and Maurer, P. (2002) *J Clin Invest* **110**, 505-513
28. Spitznagel, L., Nitsche, D. P., Paulsson, M., Maurer, P., and Zaucke, F. (2004) *Biochem J* **377**, 479-487
29. Mayer, U., Nischt, R., Poschl, E., Mann, K., Fukuda, K., Gerl, M., Yamada, Y., and Timpl, R. (1993) *EMBO J* **12**, 1879-1885
30. Mayer, U., Poschl, E., Gerecke, D. R., Wagman, D. W., Burgeson, R. E., and Timpl, R. (2004) *Biochem J* **377**, 479-487
31. Walter, P., Gilmore, R., and Blobel, G. (1984) *Cell* **38**, 5-8
32. Walter, P., and Johnson, A. E. (1994) *Annu Rev Cell Biol* **10**, 87-119
33. von Heijne, G. (1985) *J Mol Biol* **184**, 99-105
34. Perlman, D., and Halvorson, H. O. (1983) *J Mol Biol* **167**, 391-409
35. Martoglio, B., and Dobberstein, B. (1998) *Trends Cell Biol* **8**, 410-415
36. Rutkowski, D. T., Lingappa, V. R., and Hegde, R. S. (2001) *Proc Natl Acad Sci U S A* **98**, 7823-7828
37. Kim, S. J., Mitra, D., Salerno, J. R., and Hegde, R. S. (2002) *Dev Cell* **2**, 207-217
38. Martoglio, B. (2003) *Biochem Soc Trans* **31**, 1243-1247
39. Rutkowski, D. T., Ott, C. M., Polansky, J. R., and Lingappa, V. R. (2003) *J Biol Chem* **278**, 30365-30372
40. Helenius, A., and Aebi, M. (2001) *Science* **291**, 2364-2369
41. Loughlin, J., Irven, C., Mustafa, Z., Briggs, M. D., Carr, A., Lynch, S. A., Knowlton, R. G., Cohn, D. H., and Sykes, B. (1998) *Hum Mutat Suppl* **1**, S10-17
42. Cho, J. Y., Grant, T. D., Lunstrum, G. P., and Horton, W. A. (2001) *Am J Med Genet* **106**, 251-253
43. Bird, P., Gething, M. J., and Sambrook, J. (1990) *J Biol Chem* **265**, 8420-8425
44. Kyte, J., and Doolittle, R. F. (1982) *J Mol Biol* **157**, 105-132
45. Hashimoto, Y., Tomiyama, T., Yamano, Y., and Mori, H. (2003) *Am J Pathol* **163**, 101-110
46. Svensson, L., Aszodi, A., Heinegard, D., Hunziker, E. B., Reinhold, F. P., Fassler, R., and Oldberg, A. (2002) *Mol Cell Biol* **22**, 4366-4371
47. Kosfeld, M. D., and Frazier, W. A. (1992) *J Biol Chem* **267**, 16230-16236
48. Zheng, T., and Nicchitta, C. V. (1999) *J Biol Chem* **274**, 36623-36630
49. Li, Y., Bergeron, J. J., Luo, L., Ou, W. J., Thomas, D. Y., and Kang, C. Y. (1996) *Proc Natl Acad Sci U S A* **93**, 9606-9611
50. Vertel, B. M., Velasco, A., LaFrance, S., Walters, L., and Kaczman-Daniel, K. (1989) *J Cell Biol* **109**, 1827-1836
51. Froeschke, M., Basler, M., Groettrup, M., and Dobberstein, B. (2003) *J Biol Chem* **278**, 41914-41920
52. Lyko, F., Martoglio, B., Jungnickel, B., Rapoport, T. A., and Dobberstein, B. (1995) *J Biol Chem* **270**, 19873-19878
53. Martoglio, B., Graf, R., and Dobberstein, B. (1997) *EMBO J* **16**, 6636-6645
54. Chen, H., Deere, M., Hecht, J. T., and Lawler, J. (2000) *J Biol Chem* **275**, 26538-26544
55. Hou, J., Putkey, J. A., and Hecht, J. T. (2000) *Cell Calcium* **27**, 309-314
56. Brostrom, M. A., and Brostrom, C. O. (2003) *Cell Calcium* **34**, 345-363
FOOTNOTES

We thank Ulrike Mayer for providing the pCEP/γ2III4 vector. We are grateful to Lynn Sakai for many fruitful discussions. We thank Patti Kloss and Kerry Maddox for dedicated sequencing and Nicholas P. Morris for critical reading of this manuscript. This work was supported by a research fellowship (PH) and research grants (DRK, HPB, WAH) from the Shriners Hospitals for Children.

1The abbreviations used are: COMP, cartilage oligomeric matrix protein; HEK-293, Human Embryonic Kidney-293 cell line; MED, multiple epiphyseal dysplasia; PSACH, Pseudoachondroplasia; RCS, rat chondrosarcoma; ER, rough endoplasmic reticulum; SP, signal peptide; WT, wild type.

FIGURE LEGENDS

Figure 1. Graphical representation of a COMP monomer and hydropathy analysis of the COMP and BM40 signal peptides. (A) The domain structure of a COMP monomer showing the approximate positions of the PSACH (del D469) and MED (del RG367-368) causing mutations studied in this paper. (B) The hydropathy profiles of the COMP and BM40 signal peptides (SP) were determined by the Kyte-Doolittle algorithm using a 9-residue window. Analyses were performed using the ‘Protscale’ tool on the expasy server on the World Wide Web. The single letter amino acid sequence of each signal peptide is shown. Residues in bold and underlined represent the mature amino terminus of each protein following signal peptide cleavage. In each case italicized residues denote the start of the mature COMP protein sequence.

Figure 2. Analysis of COMP synthesis and secretion by transfected HEK-293 cells. (A) Equal amounts of COMP constructs as indicated and empty vector (Mock) were transiently transfected into HEK-293 cells. Culture medium and cell lysates were analyzed by western blot using an anti His-tag and an anti GAPDH antibody to demonstrate equal loading of lanes. The doublet in the media samples results from proteolysis within the amino-terminal domain of COMP (P. Holden unpublished results). (B) The experiment was repeated in triplicate and band densities were quantified. The ratio of COMP in the medium to COMP in the cell lysate was then calculated for each construct and the average value plotted. SP = Signal Peptide.

Figure 3. Localization of His-tagged COMP in transiently transfected HEK-293 cells and colocalization with the ER marker DsRed2-ER. HEK-293 cells expressing wild type (WT) or mutant (del D and del RG) COMP targeted by the BM40 (left panel) or COMP (middle panel) signal peptide and HEK-293 cells stably expressing the pDsRed2-ER marker and transiently expressing WT or mutant (del D and del RG) COMP targeted by the COMP signal peptide (right panel) were processed for immuno-fluorescence and viewed using a Leica TCS SP2 confocal microscope system. The images in the left and middle panels represent a maximum projection of slices taken through a whole cell at 0.2 µm intervals. The images in the right panel are single slices of cells. Scale bar = 4 µm.

Figure 4. Ultrastructural analysis of HEK-293 cells expressing COMP by TEM. HEK-293 cells expressing wild type (WT) or mutant (del D and del RG) COMP targeted by the COMP signal peptide were processed for TEM. Images revealed structures resembling dilated rER in cells expressing mutant COMP. Scale bars in the left and right panel are 2 µm and 500 nm respectively.

Figure 5. Pulse-chase analysis of COMP secretion in stably transfected HEK-293 cells. (A) HEK-293 cells stably expressing COMP as indicated (left) were pulsed for ½ hour with medium containing radio-labelled cysteine and methionine and chased for 24 hours in cold medium. Culture media and cell lysate samples were subjected to SDS-PAGE under reducing conditions followed by fixing and drying of the
gels. Gels were then exposed to a phosphor-imaging screen overnight and the screens read the next day using the STORM system. (B) Secreted COMP (Media) at each time point was quantified using the Image-Quant analysis program and the results plotted.

**Figure 6.** Extended culture of stably transfected HEK-293 cells. (A) Triplicate sets of HEK-293 cells stably expressing COMP as indicated (Top) were cultured for 6 days. Culture medium was harvested and cell lysates prepared and both were analyzed by western blot. Signal was generated using the ECF system and detected by the STORM instrument. (B) COMP in the cell lysates and in the culture medium for each construct in triplicate was quantified using the Image-Quant analysis program. In each case the total value for wild type COMP targeted by the COMP signal peptide was assigned a value of 1 and the values for the remaining constructs were adjusted relatively.

**Figure 7.** Analysis of hetero-oligomer formation in HEK-293 cells co-transfected with wild type and mutant COMP DNA and the effect of the BM40 signal peptide on COMP secretion in RCS cells. (A) Wild type (WT) and mutant (del D or del RG) COMP constructs containing either a C-terminal His-tag or a C-terminal FLAG-tag as indicated (Top) were co-transfected transiently into HEK-293 cells. Culture medium was harvested 24 hours post transfection and analyzed by western blot. (B) Media from each transfection was then purified using Nickel affinity resin and the purified protein subjected to 3-8% SDS-PAGE under non-reducing conditions followed by western blot analysis using an anti-FLAG tag antibody. (C) Equal amounts of COMP constructs as indicated were transiently transfected into RCS cells. Culture medium and cell lysates were analyzed by western blot 72 hours post transfection using an anti His-tag antibody.
Figure 1
Figure 2

A

DNA

|       | COMP SP | BM40 SP |
|-------|---------|---------|
| Mock  | ![](DNA_Mock) | ![](DNA_Mock) |
| WT    | ![](DNA_WT)  | ![](DNA_WT)  |
| del D | ![](DNA_delD) | ![](DNA_delD) |
| del RG| ![](DNA_delRG) | ![](DNA_delRG) |

Cell lysates

|       | COMP SP | BM40 SP |
|-------|---------|---------|
| His   | ![](Cell_Lysates_His) | ![](Cell_Lysates_His) |
| GAPDH | ![](Cell_Lysates_GAPDH) | ![](Cell_Lysates_GAPDH) |

Culture media

|       | COMP SP | BM40 SP |
|-------|---------|---------|
| His   | ![](Culture_Media_His) | ![](Culture_Media_His) |

B

![Bar graph](BarGraph.png)
Figure 3
Figure 5
Figure 7
Secretion of cartilage oligomeric matrix protein is affected by the signal peptide
Paul Holden, Douglas R. Keene, Gregory P. Lunstrum, Hans Peter Bachinger and William
A. Horton

*J. Biol. Chem.* published online March 3, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M411716200

Alerts:  
- When this article is cited  
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts