Collagen X Chains Harboring Schmid Meta physeal Chondrodysplasia NC1 Domain Mutations Are Selectively Retained and Degraded in Stably Transfected Cells*

Richard Wilson, Susanna Freddi, and John F. Bateman‡

From the Cell & Matrix Biology Research Unit, Department of Paediatrics, University of Melbourne and the Murdoch Childrens Research Institute, Royal Children’s Hospital, Parkville, Victoria 3052, Australia

Collagen X is a short chain, homotrimeric collagen expressed specifically by hypertrophic chondrocytes during endochondral bone formation and growth. Although the exact role of collagen X remains unresolved, mutations in the COL10A1 gene disrupt growth plate function and result in Schmid metaphyseal chondrodysplasia (SMCD). With the exception of two mutations that impair signal peptide cleavage during α1(X) chain biosynthesis, SMCD mutations are clustered within the carboxyl-terminal NC1 domain. The formation of stable NC1 domain trimers is a critical stage in collagen X assembly, suggesting that mutations within this domain may result in subunit mis-folding or reduce trimer stability. When expressed in transiently transfected cells, α1(X) chains containing SMCD mutations were unstable and presumed to be degraded intracellularly. More recently, in vitro studies have shown that certain missense mutations may exert a dominant negative effect on α1(X) chain assembly by formation of mutant homotrimers and normal-mutant heterotrimers. In contrast, analysis of cartilage tissue from two SMCD patients revealed that the truncated mutant message was fully degraded, resulting in 50% reduction of functional collagen X within the growth plate. Therefore, in the absence of data that conclusively demonstrates the full cellular response to mutant collagen X chains, the molecular mechanisms underlying SMCD remain controversial. To address this, we closely examined the effect of two NC1 domain mutations, one frameshift mutation (1963del10) and one missense mutation (Y598D), using both semi-permeabilized cell and stable cell transfection expression systems. Although able to assemble to a limited extent in both systems, we show that, in intact cells, collagen X chains harboring both SMCD mutations did not evade quality control mechanisms within the secretory pathway and were degraded intracellularly. Furthermore, co-expression of wild-type and mutant chains in stable transfected cells demonstrated that, although wild-type chains were secreted, mutant chains were largely excluded from hetero-trimer formation. Our data indicate, therefore, that the predominant effect of the NC1 mutations Y598D and 1963del10 is a reduction in the amount of functional collagen X within the growth cartilage extracellular matrix.

Collagen X is a short-chain collagen expressed specifically at sites of endochondral ossification during normal skeletal development and under conditions that involve new bone growth, such as fracture repair or osteoarthritis (1–3). Although the precise role of collagen X remains ill-defined, it is proposed to provide structural support within the extracellular matrix of the epiphyseal growth plate during the transition from cartilage to bone (4). Collagen X is a homotrimer of three α1(X) chains (M₉ 59,000), each composed of a collagenous domain (COL1) flanked by the carboxyl and amino termini by non-collagenous extensions, the NC1 and NC2 domains. Unlike the fibrillar collagens, the NC1 and NC2 domains of collagen X are not removed by proteolytic processing and are thought to direct supramolecular assembly of collagen X molecules into a hexagonal lattice that forms mat-like structures within the matrix (5). Collagen X has also been observed as fine pericellular filaments associated with collagen II fibrils within the hypertrophic cartilage matrix (6) leading to the hypothesis that functional roles for collagen X may differ according to the type of aggregate formed (7).

Given the restricted localization of collagen X to growth plate cartilage, it is not surprising that COL10A1 mutations result in the cartilage defect, Schmid metaphyseal chondrodysplasia (SMCD). However, the precise consequences of mutations on collagen X biosynthesis, secretion, and supramolecular assembly and their impact on SMCD molecular pathology have not been fully elucidated and remain controversial. SMCD is an autosomal dominant disorder of the osseous skeleton resulting in a relatively mild phenotype associated with growth plate abnormalities (8–10). SMCD mutations characterized to date include single base substitutions, nonsense mutations, and deletions resulting in premature termination (for review, see Ref. 10). Apart from two missense mutations, which affect the putative signal peptide cleavage site (11), the remaining SMCD mutations identified are localized within the NC1 domain. The association of carboxyl-terminal domains is an important step in assembly of fibrillar procollagens and a potential site for NC1-NC1 interaction was identified based upon alignment of collagen X and fibrillar procollagen carboxyl-terminal amino acid sequences (12). This conserved aromatic motif (leucine 589–601) is indeed critical for the interaction of NC1 domains in vitro (13).

Based on the observation that SMCD mutations prevented
the formation of SDS-stable trimers, it was initially proposed that SMCD is caused by exclusion of mutant chains from a1(X) assembly, resulting in a 50% reduction in functional collagen X within the growth plate (14). In two cases, analysis of SMCD patient cartilage has shown that the presence of a premature termination codon results in nonsense-mediated decay of the mRNA derived from the mutant allele, supporting the haploinsufficiency model (15).3 Missense mutations, however, are unlikely to reduce mRNA stability, and studies have shown that a1(X) chains harboring certain point mutations can form stable homotrimers and co-assemble with the normal chain when expressed in semi-permeabilized cells (16). An in vitro competition-based assay that is not dependent on the formation of SDS-stable trimers also showed that SMCD mutant chains could reduce the efficiency of normal a1(X) chain assembly (13).

Thus the model emerging from these studies is that SMCD missense mutations exert a dominant negative effect on collagen X assembly. However, in vitro expression systems may not accurately reproduce the full cellular response to mutant collagen X mRNA or mutant a1(X) chains. Indeed, expression of collagen X in transiently transfected mammalian cells demonstrated that wild-type a1(X) chains were secreted efficiently whereas SMCD mutant a1(X) chains were present in only trace amounts within the media (17). It is not clear whether lack of mutant chain secretion was due to poor transcription levels of the mutant a1(X) constructs or rapid intracellular degradation of a1(X) chains.

In this study we have used two expression systems to evaluate the effect of the SMCD NC1 point mutation, Y598D, and a frameshift mutation (18) resulting from a 10-bp deletion within the growth plate (14). In two cases, analysis of SMCD 1(X) chains, expressed in semi-permeabilized HT 1080 cells, assembled with similar efficiency to the normal chain when analyzed under low SDS concentrations (16). However, in vitro translated human Y598D trimeric chains have markedly reduced stability in comparison to the wild-type NC1 trimer, when analyzed under similar conditions (20). Here we find that human a1(X) chains carrying the Y598D and NC1(X) mutations assemble less efficiently than the wild-type chain when expressed in semi-permeabilized cells. A similar pattern emerged when Y598D and NC1(X) constructs were stably transfected into human bone (SaOS-2) cells. Although expressed at the mRNA level, mutant chains were retained within the cell and degraded whereas normal chains were secreted. To assess whether mutant chains could be incorporated into stable heterotrimers, we generated co-transfected cell lines expressing wild-type a1(X) chains together with either Y598D or NC1(X) a1(X) chains. Our results show that only trace amounts of mutant collagen X chains could be detected in the media, indicating that the predominant effect of these mutations is a 50% reduction in functional collagen X secretion.

EXPERIMENTAL PROCEDURES

Construction of Wild-type and Mutant Collagen X Recombinant Plasmids for in Vitro Expression.—The production of full-length human a1(X) cDNA (aPTM1-h10wt) and engineering of the SMCD mutants used in this study have been described previously. The wild-type a1(X) sequence was cloned into the T7-driven expression vector, pTM1, to enable transcription of mRNA in vitro (14). Two SMCD mutations, 1963del10 (18) and Y598D (9), were generated by strand overlap exten-

3 J. F. Bateman, S. Freddi, R. Savarirayan, and G. Nattrass, unpublished data.
4 The base pairs in the COL1A1 cDNA sequence are numbered from the transcription start site (35).

sion PCR using specific oligonucleotide primer sets as described previously (17). The 10-bp deletion at position 1963 (NC1Δ10) introduces nonsense sequence from amino acid residue 623 resulting in premature termination at residue 671. The missense mutation Y598D generates a single amino acid (tyrosine to aspartate) substitution at residue 598 in the NC1 domain.

In Vitro Transcription—Generation of full-length mRNA transcripts encoding wild-type and SMCD mutant a1(X) chains was carried out as previously described (21) using 10 μg of plasmid cDNA linearized with SalI. Transcription reactions were performed for 4 h at 30 °C using 60 units of T7 RNA polymerase (Promega). RNA was purified using an RNase-free phenol:chloroform extraction in 10-μl aliquots and stored at −70 °C (3-5 μg) or 60 aliquots containing 1 unit/μl RNasin (Promega) and 1 μl dithiothreitol.

In Vitro Translation in Semi-permeabilized HT 1080 Cells—HT 1080 fibroblasts (ATCC-CCL121, American Type Culture Collection) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% (v/v) fetal calf serum. HT 1080 cells were treated with digitonin (Calbiochem) at a final concentration of 40 μg/ml to generate semi-permeabilized cells, which were used to supplement rabbit reticulocyte lysate-based in vitro translation as previously described (22). Each translation contained 17.5 μl of Flexi-lysate (Promega), 1 μl of transcribed mRNA, 0.5 μl of 1 μM amino acids (minus methionine), 0.5 μl of 2.5 M KCl, 1 μl of 1-(10-3)M methionine (1.5 μCi/ml, ICN Pharmaceuticals Inc.), 0.25 μl of 25 mM sodium ascorbate (Sigma), and 4 μl of semi-permeabilized cells. After a 2-h incubation at 37 °C, semi-permeabilized cells were isolated by centrifugation and prepared for electrophoresis or treatment with chemotrypsin and trypsin.

Construction of Wild-type and SMCD a1(X) Expression Constructs for Stable Transfection.—For the stable expression of His6-tagged collagen X in SaOS-2 cells we generated three expression constructs (pCEP4-wt-his, pCEP4-Y598D-his, and pCEP4-NC1Δ10-his) encoding a1(X) sequences flanked at the amino termini by the BM-40 signal peptide, a 6-histidine tag, and enterokinase cleavage site. Wild-type, Y598D, and NC1Δ10 collagen X sequences in the plasmid pGEM11 were amplified using primers HX-SP (5′-GCGGGCGGCGTTTACTCCTGACGGTATA-3′) and HX6 (5′-CAGCCCGCTTTTACCTACCTCCGATA-3′) corresponding to nucleotides 56–75 and 2235–2216. PCR amplification was performed at 60 °C for 30 cycles, using 0.5 unit of Pfu polymerase (Stratagene), to generate a 2-kb fragment corresponding to the entire collagen X coding sequence lacking only the signal peptide. Additional sequences encoding NotI restriction enzyme sites were added to primers (sequence underlined) to enable subsequent cloning into the plasmid pCEP4-BM40-hisEK (23), kindly donated by Dr. Anders Aspegren, Lund University, Sweden. This plasmid is derived from pCEP4 (Invitrogen), which encodes EBNA1, a gene that allows the plasmid to be maintained episomally, and the hygromycin resistance gene, which allows selection of positive transfectants.

For the stable expression of c-Myc-tagged collagen X in SaOS-2 cells we used an expression construct (pCevCMV-wt-myc) encoding wild-type collagen X sequence flanked at the amino terminus by the BM-40 signal sequence and the c-Myc epitope. To introduce the c-Myc tag (amino acid sequence: -670 to 725) into the pCMV sequence, PCR amplified (Stratagene) and performed strand PCR using the plasmid pGEM11 containing the wild-type collagen X coding sequence lacking signal peptide as template and specific primer sets incorporating the c-myc coding sequence (underlined). The primary round of PCR were carried out using primer sets MYC-F1 (5′-AAGCTTCTTCTGCTGCGCGCTG-3′) and MYC-R1 (5′-CAGATCCCTCAGATGAGATTTTCCTGCGCCGGCTAGTACGAGGG-3′) or MYC-F2 (5′-AAGCTTCTGCTGCGCGCTG-3′) and MYC-R2 (5′-CACCAGGTTCTCTCCGCTG-3′) to generate independent fragments with overlapping sequences. Second round PCR reactions were carried out with primers MYC-F1 and MYC-R2 using 5 ng of the primary PCR products as template. All PCR amplifications were performed at 62 °C for 35 cycles, using 0.5 unit of DeepVent polymerase (New England BioLabs). The recombinant PCR product was digested with NheI and XhoI. The resulting 225-bp fragment was purified using a GeneClean kit (Qiagen) and used to replace the corresponding sequence in the pGEM7 construct encoding the full-length wild-type collagen X sequence. Finally, this plasmid was digested with NheI and XhoI to release the entire 2.2-kb collagen X sequence, which was introduced into pFceCMV (Invitrogen), an expression vector that encodes the neomycin phosphotransferase gene and conferring resistance to the antibiotic G418. Constructs were sequenced (AmpliCycle, PerkinElmer Life Sciences) to verify insertion of the c-myc sequence and to ensure insertion of the recombinant fragment in the correct reading frame.

Stable Transfection of SaOS-2 Cells.—The human osteosarcoma cell line, SaOS-2 (ATCC HTB-85, American Type Culture Collection), was
maintained in DMEM (Invitrogen) containing 10% (v/v) fetal calf se-
rum. SaOS-2 cells were grown to ~70% confluence and transfected with
the αI(X) expression constructs using FuGENE 6 transfection reagent
(Roche Molecular Biochemicals) according to the manufacturer’s pro-
col. Cells transfected with pCEP4-wt-his, pCEP4-Y593D-his, and
pCEP4-NC1Δ10-his were selected and maintained in growth medium
containing 250 μg/ml hygromycin B (Roche Molecular Biochemicals).
Cells transfected with pReCMV-wt-myc were selected in growth me-
dium containing 500 μg/ml G418 (Invitrogen) and individual G418-
resistant colonies were isolated and expanded into cell lines. One cell
line expressing wt-myc collagen X mRNA, as shown by Northern analy-
sis, was used for expression analysis and subsequent co-ex-
pression studies. Co-transfection of wt-myc cells with pCEP4-wt-his,
pCEP4-Y593D-his, and pCEP4-NC1Δ10-his was performed using the transfection protocol described above. Co-transfected cells were main-
tained in growth medium containing 500 μg/ml G418 and 250 μg/ml
hygromycin B.

Northern Blot and Hybridization Analysis—RNA was prepared from
transfected and untransfected cells cultured in individual 35-mm diam-
eter dishes at ~80% confluence, using the RNeasy extraction kit (Qia-
gen). Heat-denatured RNA (3 μg/sample) was electrophoresed on a 1%
(w/v) agarose gel containing 7% (v/v) formaldehyde, followed by capil-
lar blotting onto a nitrocellulose filter for 16 h. The filter was air-dried,
 baked under vacuum at 80 °C for 1 h, and pre-hybridized for 2 h in 25% (v/v) formamide, sheared herring sperm DNA (100 μg/ml final
concentration), Denhardt’s solution, 4 μg EDTA, and 0.1% (w/v) SDS.
A 32P-labeled probe was prepared using a 2-kb NcoI-digested product of
pTM1-h10wt (14), purified using the GeneClean kit (Qiagen). Hybrid-
ization of the probe was carried out at 42 °C overnight, and the washed
filter was exposed to autoradiography film at ~70 °C for 2 weeks.

Biopsychic Synthetic Labeling and Immunoprecipitation—Biopsychic labeling
was performed by incubation of transfected cells grown to conflui-
ence in 6-well plates with 1 ml of methionine-free, serum-free DMEM
(Invitrogen) containing 0.25 mM sodium ascorbate and 100 μCi of
L-[35S]methionine (Transtrans-label, 1032 Ci/mmol, ICN Pharmaceuticals
Inc.). When appropriate, sodium ascorbate was replaced with 0.3 mM
αα’ dipyridyl (Calbiochem). In pulse-labeling experiments, cells were pre-
treated for 1 h with 1 ml of methionine-free, serum-free DMEM,
 prior to addition of 100 μCi of L-[35S]methionine. After 1 h in labeling
media, cells were chased for a further hour in serum-free DMEM
containing excess unlabeled methionine. Where indicated, media was
supplemented with 5 μM clasto-lactacystin β-lactone (Calbiochem) or 1
mM brefeldin A (Roche Molecular Biochemicals). After removal of the
media, the cells were lysed on ice using 1 ml of ice-cold immunoprecipita-
tion buffer. Lysis buffer contained 150 mM HCl, 150 mM NaCl, 1 mM
(v/v) Triton X-100 containing 1 mM 4-(2-aminomethyl)-benzenesulfonyl
fluoride hydrochloride (AEBSF) (Roche Molecular Biochemicals) and 2
mM N-ethylmaleimide (Sigma Chemical Co.). Lysates and medium
samples were centrifuged (13,000 × g for 20 min) at 4 °C, incubated
for 1 h at 4 °C with 100 μl of protein A-Sepharose (20% (w/v) in
PBS) and centrifuged (13,000 × g for 2 min) to remove protein
A-Sepharose-bound components. Immunoprecipitation of αI(X) chains
was performed overnight at 4 °C in the presence of 10 μl of the appro-
priate antisera and 100 μl of protein A-Sepharose (20% (w/v) in
PBS). The rabbit polyclonal serum, raised to the NC1 domain of human
collagen X, was a gift from Dr. Olena Jacenko (15). Mouse monoclonal
antibodies against the e-Myc epitope (clone 9E10), and His6 epitope tags
 were from Roche Molecular Biochemicals and used at a final concen-
tration of 1 μg/ml. Immunoprecipitates were washed three times with
immunoprecipitation buffer and either prepared for SDS-PAGE analy-
sis or treatment with chymotrypsin and trypsin. Sequential Immunoprecipitations—Cell lysates and media fractions
were prepared for immunoprecipitation as described above. Primary
immunoprecipitations were carried out at 4 °C for 5 h using anti-e-Myc
or anti-His6 antibodies to isolate epitope-tagged αI(X) chains. Where
indicated, samples were analyzed directly without performing a second-
ary immunoprecipitation. Otherwise, samples were resuspended in 50
μl of immunoprecipitation buffer containing 2% (w/v) SDS and heated
to 65 °C for 10 min to elute αI(X) chains. Samples were centrifuged
briefly (13,000 × g for 30 s), and the supernatants were carefully
removed into fresh tubes and diluted to 1 ml in immunoprecipitation
buffer. Immunoprecipitation of αI(X) chains was performed overnight
at 4 °C using 100 μl of 20% (w/v) protein A-Sepharose in the presence or
absence of anti-Myc or anti-His6 secondary antibodies, used at a final
concentration of 2 μg/ml.
Chymotrypsin/Trypsin Digestion—Protease treatment of semi-per-
meabilized cell translation products was carried out in 50 μl of CT/T
digestion buffer (50 mM Tris- HCl, pH 7.4, containing 150 mM NaCl and
10 mM EDTA) supplemented with 0.5% (w/v) Triton X-100 to solubilize
cells. Immunoprecipitates were resuspended in 50 μl of digestion buffer
without Triton X-100. Samples were incubated with a combination of
tryptsin (100 μg/ml) and chymotrypsin (250 μg/ml) for 2 min at 25 °C.
Digests were stopped by addition of soybean trypsin inhibitor (Sigma)
at a final concentration of 5 μg/ml and two volumes of boiling SDS-
PAGE sample buffer.
SDS-PAGE and Immunoblotting—Samples were prepared for elec-
trophoresis by boiling for 5 min in an equal volume of SDS-PAGE sample
buffer (50 mM Tris/ HCl, pH 6.8, 2% (w/v) SDS, 20% glycerol, and
0.025% (w/v) bromphenol blue) unless otherwise stated. All samples
were resolved through 7.5% SDS-PAGE gels, and labeled proteins
were visualized by autoradiography, fluorography, or phosphorimaging (Mol-
ecular Dynamics, Storm) where indicated (see “Results”). Samples for
immunoblotting were transferred to nitrocellulose and incubated for 1 h
in PBS containing 0.1% Tween 20 in the presence of 5% (w/v) milk
protein. The filter was probed with anti-KDEL monoclonal anti-
body, and primary antibodies were detected using rabbit anti-mouse
IgG-horseradish peroxidase conjugate. Blots were developed by chemi-
luminescence (ECL, Amersham Biosciences, Inc.) according to the man-
ufacturer’s protocol.

RESULTS

Human Collagen X Chains Harboring SMCD Mutant Collagen X Chains
Y598D and NC1Δ10 Assemble Less Efficiently Than Wild-type
Chains When Expressed in Semi-permeabilized Cells—In this study
we used an in vitro translation system supplemented with semi-permeabilized human fibroblasts (HT 1080 cells) as a starting point to analyze the effect of SMCD mutations on assembly of the full-length human αI(X) chain. The αI(X) cDNAs encoding one SMCD point mutation, Y598D, and one frameshift mutation, NC1Δ10, were transcribed in vitro and translated in the presence of rabbit reticulocyte lysate supplemented with semi-permeabilized HT 1080 cells. Translation products were prepared for electrophoresis without heating and using 0.5% (w/v) SDS to detect trimers, which may disso-
ciate under more harsh conditions (Fig. 1). A high proportion of wild-type αI(X) chains formed SDS-stable trimers, whereas both NC1 Y598D and NC1Δ10 mutant chains migrated essentially as monomers (lanes 1–3). This result indicates that the

FIG. 1. Analysis of wild-type and mutant αI(X) chain assembly in semi-permeabilized cells. Collagen X mRNA encoding wild-type (lanes 1 and 4) or SMCD mutant collagen X (lanes 2, 3, 5, and 6) was translated in rabbit reticulocyte lysate supplemented with semi-perme-
abilized HT 1080 cells for 60 min at 30 °C. Following translation, semi-permeabilized cells were separated from the mixture by centrifugation, and translated products were either incubated in 0.5% (w/v) SDS for 5 min at 25 °C (lanes 1–3) or treated with chymotrypsin and trypsin (CT/T; lanes 4–6) followed by separation through a 7.5% SDS-
PAGE gel. Proteins were visualized by fluorography, and quantitation was performed by phosphorimaging analysis. The migration positions of helical domains (αI(X)h), monomeric (αI(X)), and trimeric (αI(X)3) chains are indicated.
ability of the NC1 domains to form SDS-stable trimers is severely compromised by both SMCD mutations. Despite this apparent lack of electrophoretic stability, weak or transient association of mutant \(\alpha 1(X)\) chains within the endoplasmic reticulum may result in triple-helix nucleation and subsequent propagation. Therefore, normal and mutant \(\alpha 1(X)\) chains were synthesized in semi-permeabilized cells in the presence of ascorbate, and translation products were treated with chymotrypsin and trypsin to probe formation of correctly folded triple helices. As expected, a high proportion of wild-type \(\alpha 1(X)\) chains assembled into correctly aligned triple helices as evidenced by a protease-resistant band corresponding to the collagenous domain (lane 4). In contrast, a much smaller fraction of the Y598D and NC1Δ10 chains synthesized was protease resistant (lanes 5 and 6). Together, these results demonstrate, using established assays, that the SMCD-causing mutations Y598D and NC1Δ10 significantly reduce the efficiency of human collagen X assembly in semi-permeabilized cells.

**Mutant Collagen X Chains in Stably Transfected SaOS-2 Cells Are Expressed at the mRNA Level but the Protein Is Not Secreted**—The chaperones and enzymes required for correct post-translational processing of collagens are present within the endoplasmic reticulum of semi-permeabilized HT1080 cells, allowing the initial stages of collagen folding and assembly to be reconstituted in vitro (16, 24, 25). However, the full cellular response to mutant \(\alpha 1(X)\) chains can only be assessed within the context of intracellular transport and secretion quality control. Data concerning the secretion of mutant collagen X homo- or heterotrimers is lacking due to the low expression level of SMCD mutants in transiently transfected cells (17). To overcome this, we generated stably transfected SaOS-2 cells expressing the wild-type human \(\alpha 1(X)\) chain and the NC1 SMCD mutants Y598D and NC1Δ10. As part of our strategy for analysis of normal and mutant \(\alpha 1(X)\) heterotrimer formation, we cloned the wild-type \(\alpha 1(X)\) cDNA and mutant \(\alpha 1(X)\) cDNAs into different vectors containing different antibiotic resistance markers, allowing us to select and culture cells co-expressing both wild-type and mutant chains.

To study the formation of wild-type and mutant collagen X homotrimers, cell lines expressing wild-type, NC1 Y598D, and NC1Δ10 \(\alpha 1(X)\) chains were established (see “Experimental Procedures” for details). SaOS-2 cells were transfected with either wild-type collagen X cDNA in pRc/CMV-BM40-myc (wt-myc) or mutant collagen X cDNAs in pCEP4-BM40-hisEK (Y598D-his and NC1Δ10-his, Fig. 2). Northern analysis of RNA from wt-myc, Y598D-his, and NC1Δ10-his transfected SaOS-2 cells confirmed the expression of wild-type and mutant collagen X mRNA (Fig. 3, lanes 1–3) whereas collagen X was not expressed in untransfected cells (lane 4).

To assess whether wild-type and mutant \(\alpha 1(X)\) chains are secreted, transfected cells and control (untransfected) SaOS-2 cells were biosynthetically labeled with \[^{14}S\]methionine. Wild-type (wt-myc) and mutant (Y598D-His and NC1Δ10-His) \(\alpha 1(X)\) chains were recovered from cell and media fractions by immunoprecipitation using anti-c-Myc and anti-His\(_6\) antibodies (Fig. 4). The cell and media fractions of untransfected cells were incubated with antibodies raised to the human \(\alpha 1(X)\) NC1 domain. Collagen X chains were detected within the cell fractions of the three transfected cell lines but absent from untransfected SaOS-2 cells, consistent with the mRNA expression pattern (Fig. 3). As samples were denatured prior to electrophoresis, collagen X trimers were not observed. Strikingly, although normal \(\alpha 1(X)\) chains were secreted (lane 6), mutant chains were not detected in the media fraction (lanes 7 and 8). Similar results were obtained when anti-collagen X antibodies were used instead of anti-c-Myc or anti-His\(_6\) antibodies, indicating that detection of normal and mutant \(\alpha 1(X)\) chains was not biased by the use of two different epitope tags (data not shown). Considering the high level of Y598D and NC1Δ10 mRNA expression relative to the wild-type collagen X mRNA (Fig. 3) and the lack of accumulated intracellular material, these data strongly suggest that mutant \(\alpha 1(X)\) chains are degraded via an intracellular pathway.

**Mutant \(\alpha 1(X)\) Chains Are Degraded via Multiple Intracellular Pathways**—To investigate the mechanism by which mutant \(\alpha 1(X)\) chains are degraded, cells expressing either wild-type or mutant collagen X chains were biosynthetically labeled in the presence of the proteasomal inhibitor clasto-lactacystin \(\beta\)-lactone or brefeldin A, a reagent that prevents intracellular traffic to the lysosomal/endosomal compartments. Cells were pre-
treated for 1 h with or without inhibitors, pulse-labeled for 1 h then incubated in chase medium (see “Experimental Procedures”). In the absence of both inhibitors, wild-type (lane 1) but not mutant (lanes 5 and 9) α1(X) chains were detected in the media fraction. As expected, inclusion of brefeldin A abolished secretion of wild-type α1(X) chains (lanes 3 and 4), resulting in accumulation of material in the intracellular fraction. Significantly higher levels of intracellular Y598D and NC1Δ10 chains were detected when cells were treated with either clasto-lactacystin β-lactone (lanes 6 and 10, respectively) or brefeldin A (lanes 7 and 11, respectively). Furthermore, incubation with a combination of both inhibitors resulted in enhanced protection of mutant α1(X) chains in the cellular fraction (lanes 8 and 12). These data suggest that mutant chains are degraded via both proteasomal and vesicular transport-dependent pathways.

Mutant α1(X) Chains That Are Not Degraded Can Form Correctly Folded Helical Domains—Our results demonstrate that Y598D and NC1Δ10 α1(X) chains are not secreted but are degraded via lactacystin- and brefeldin A-sensitive pathways. However, at the end of overnight labeling (Fig. 4) and pulse-labeling (Fig. 5) a minor fraction of the retained mutant chains remain undegraded. To analyze the folding status of these chains, transfected SaOS-2 cells were metabolically labeled overnight either in the presence of 0.25 mM ascorbate to permit hydroxylation, or in the presence of 0.3 mM α-acid glycoprotein, which effectively inhibits hydroxylation and triple helix formation (26). Cell lysates were incubated with antibodies raised to collagen X and immunoprecipitates were analyzed either directly or after digestion with chymotrypsin and trypsin to probe the formation of correctly aligned triple helices. Collagen X chains synthesized in the absence of α-acid glycoprotein (Fig. 6a, lanes 1, 3, and 5) had reduced electrophoretic mobility compared with α1(X) chains synthesized in the presence of α-acid glycoprotein (lanes 2, 4, and 6), evidence that α-acid glycoprotein prevents α1(X) chain hydroxylation. Accordingly, chains synthesized in the absence of α-acid glycoprotein had formed protease-resistant helical domains (Fig. 6b, lanes 1, 3, and 5) whereas unhydroxylated chains were degraded by protease treatment (lanes 2, 4, and 6). As shown with the semi-permeabilized cell system, Y598D and NC1Δ10 chains are capable of triple helix formation (Fig. 1). However, in intact cells it is clear that only wild-type chains are secretion-competent (Fig. 6c, lane 1), indicating that mutant chains are recognized as non-native within the endoplasmic reticulum.

Retained α1(X) Chains Are Associated with PDI/P4-H—Two endoplasmic reticulum resident proteins, Hsp47 and protein disulfide isomerase (PDI), have been identified as candidates to “chaperone” collagen X during biosynthesis (16, 27). PDI has been proposed to prevent assembly of trimers into higher-ordered structures within the endoplasmic reticulum and also binds transiently to collagen X as the β subunit of P4-H during the hydroxylation of α1(X) chains (16, 27). Our results show clearly that mutant α1(X) chains are selectively retained within the endoplasmic reticulum of stable transfectants. Therefore, we attempted to identify interactions between collagen X and any endoplasmic reticulum resident proteins using co-immunoprecipitation. Control (untransfected) and SaOS-2 cells transfected with wild-type, Y598D, and NC1Δ10 constructs were incubated overnight in the presence of 0.25 mM ascorbate to allow α1(X) chain hydroxylation and folding. Cell lysates were incubated under native conditions, without addition of chemical cross-linkers, with anti-collagen X antibodies to isolate α1(X) chains and any stably associated proteins. Immunoprecipitates were denatured, resolved by SDS-PAGE, and proteins were transferred to nitrocellulose. The filter was probed with an anti-KDEL monoclonal antibody that recognizes proteins with the carboxyl-terminal KDEL endoplasmic reticulum retention motif. A protein band with the molecular weight of PDI was co-precipitated specifically with Y598D and NC1Δ10 chains (Fig. 7, lanes 3 and 4) but not with wild-type α1(X) chains (lane 2). Previous studies using bovine α1(X) chains synthesized in semi-permeabilized cells showed
an equal level of binding of PDI to wild-type and mutant α1(X) chains (16). However, we have shown here that the formation of stable trimers and correctly aligned triple helices by human α1(X) chains is compromised by the Y598D and NC1Δ10 mutations. Our results, therefore, are consistent with a chaperone function for PDI, acting independently or as a subunit of P4-H.

When co-expressed with wild-type α1(X) chains only trace levels of Y598D chains are secreted—When expressed alone, the SMCD mutants Y598D and NC1Δ10 are unable to assemble into secretion-competent heterotrimers. However, as SMCD is a heterozygous disorder, it was important to assess whether wild-type and SMCD mutant chains could form stable heterotrimers that undergo secretion. To analyze normal-mutant α1(X) chain heterotrimer formation we generated co-transfected cell lines expressing c-Myc-tagged wild-type α1(X) chains together with either His6-tagged Y598D or His6-tagged NC1Δ10 chains (wt-myc/Y598D-his cells and wt-myc/NC1Δ10-his cells, respectively; see “Experimental Procedures” for details). As a positive control for co-assembly of chains carrying the two epitope tags, we generated a third co-transfected cell line expressing both wt-Myc and wt-His tagged chains (wt-myc/wt-his cells). Co-transfected SaOS cells (wt-myc/wt-his) were biosynthetically labeled overnight, and cell and media fractions were incubated with either anti-c-Myc or anti-His6 antibodies (Fig. 8). After washing, immune complexes were either analyzed directly (lanes 1, 2, 5, and 6) or denatured and subjected to a second round of immunoprecipitation using the reciprocal antibodies (lanes 3, 4, 7, and 8). As expected, wt chains carrying both His6 and c-Myc epitopes were secreted into the medium (lanes 5 and 6). In cell and media fractions, sequential immunoprecipitation demonstrated co-assembly of wt-Myc and wt-His chains. Our results show that, after denaturation of immune complexes, the anti-c-Myc antibody was more efficient in the secondary immunoprecipitation (lanes 4 and 8) than the anti-His6, antibody (lanes 3 and 7). Therefore, in subsequent co-assembly experiments, samples were incubated first with anti-His6 antibodies to isolate mutant chains and secondly with anti-c-Myc antibodies.

**Fig. 6.** Wild-type and SMCD mutant chain triple helix formation in transfected SaOS-2 cells. SaOS-2 cells transfected with wild-type (WT-myc) and mutant (Y598D-his and NC1Δ10-his) collagen X were biosynthetically labeled overnight with L-[35S]methionine as described (“Experimental Procedures”). Media fractions from control (untransfected) and transfected SaOS cells were incubated with polyclonal antibodies raised to collagen X. Wild-type and mutant α1(X) chains were analyzed separately by the horseradish peroxidase-secondary antibody conjugate are detected as protein smears at ~50 kDa (indicated by asterisk). A protein band with an approximate molecular mass of 60 kDa is indicated (PDI).

**Fig. 7.** Co-precipitation of mutant α1(X) chains with PDI. SaOS-2 cells transfected with wild-type (WT-myc) and mutant (Y598D-his and NC1Δ10-his) collagen X and control (untransfected) cells were incubated overnight in DMEM containing 0.25 mM dithiothreitol prior to separation on a 7.5% SDS-polyacrylamide gel, and radiolabeled proteins were visualized by fluorography. The migration positions of α1(X) monomers (α1(X)m) and protease-resistant helical domains (α1(X)h) are indicated.

**Fig. 8.** Co-expression and sequential immunoprecipitation of c-myc- and his6-tagged wild-type α1(X) chains in co-transfected SaOS-2 cells. Co-transfected cells expressing wt-Myc and wt-His α1(X) chains (wt-myc/wt-his cells) were biosynthetically labeled overnight with L-[35S]methionine as described (“Experimental Procedures”). All immune complexes were denatured in SDS-PAGE sample buffer containing 2% (w/v) SDS, prior to separation on a 7.5% SDS-polyacrylamide gel, and radiolabeled proteins were visualized by fluorography.
Immunoprecipitates were either analyzed directly (lanes 1 and 6) or after denaturation and subsequent incubation overnight with secondary antibodies as indicated (lanes 4, 5, 9, and 10). Duplicate samples were incubated under the same conditions in the absence of secondary antibodies (-Ab). In addition to co-transfected cells, SaOS-2 cells expressing only wt-Myc α1(X) chains were biosynthetically labeled (WT-myc). Media samples and cell lysates were incubated for 5 h with antibodies specific for c-Myc (m) or immunoprecipitates were analyzed (lanes 1 and 6). All immune complexes were denatured in SDS-PAGE sample buffer containing 2% (w/v) SDS, prior to electrophoresis on a 7.5% SDS-polyacrylamide gel, and radiolabeled proteins were visualized by fluorography.

To assess normal-mutant α1(X) co-assembly, co-transfected SaOS cells (wt-myc/Y598D-his and wt-myc/NC1Δ10-his) were biosynthetically labeled overnight. Cell and media fractions were incubated with either anti-c-Myc or anti-His6 antibodies and either analyzed directly or after secondary immunoprecipitation. Wild-type and mutant chains were detected in the intracellular fractions of both co-transfected cell lines (Fig. 9, a and b; lanes 2 and 3). To analyze heterotrimer formation, immune complexes containing Y598D-His or NC1Δ10-His α1(X) chains were denatured and re-precipitated with anti-c-Myc antibodies. In contrast to the wt-myc/wt-his cells (Fig. 8), after primary immunoprecipitation with anti-His6 antibodies, little or no wt-Myc chains could be recovered in the secondary immunoprecipitation (Fig. 9, a and b; lane 4). Analysis of the media showed that, in comparison to the level of wild-type chains, very little Y598D-His material was secreted (Fig. 9a, lane 8) and NC1Δ10-His chains were barely detectable in the media fraction (Fig. 9b, lane 8). In wt-myc/Y598D-his cells, a fraction of the α1(X) chains recovered using anti-His6 antibodies could be re-precipitated with anti-c-Myc antibodies (Fig. 9a, lane 9) indicating that the trace amounts of Y598D-His chains present in the media are secreted as heterotrimers. In both experiments we did not observe any significant reduction in the level of wt-Myc chains secreted into the media by co-transfected cells, compared with the original cell line expressing only wt-Myc α1(X) chains (compare lanes 6 and 7, panels a and b). Together, these results demonstrate that mutant α1(X) chains are largely excluded from heterotrimer formation. However, a very minor fraction of Y598D chains are co-assembled with wild-type chains within the cell, resulting in secretion of trace levels of normal-mutant heterotrimers.

**DISCUSSION**

In this study we have employed two expression systems to analyze the effect of SMCD mutations on the assembly and secretion of human collagen X. The ability of NC1 domains carrying point mutations to form stable homotrimers and interact with wild-type α1(X) chains has been demonstrated previously in several studies (13, 16). Using the semi-permeabilized cell system, we expressed normal and SMCD α1(X) chains to evaluate the effect of one point mutation (Y598D) and one frameshift mutation (NC1Δ10) on homotrimer and triple helix formation. Interestingly, the NC1Δ10 chains assembled poorly compared with the Y598D chains, consistent with the proposal that frameshifts may cause a more severe effect on the protein-protein interactions involved in NC1 trimer assembly (17). Comparison of the data from our study and that of McLaughlin et al. (16), shows that both wild-type and Y598D human α1(X) chains formed a lower proportion of helical material than the respective bovine homologues. One explanation for this difference is the presence of two cysteine residues within the helical domain of the bovine α1(X) chain, which would contribute to the stability of the triple helix during folding. Another property of the human and bovine α1(X) chains synthesized in this system that differs significantly is the relative stability of the homotrimers in low concentrations of SDS. Although bovine Y598D and wild-type chains formed SDS-stable homotrimers with similar efficiency, we found that the stability of human α1(X) trimers was dramatically reduced by the Y598D mutation. Previous results have shown that the human NC1 domain carrying the Y598D mutation can form SDS-stable trimers (20), although these trimers were more...
sensitive to denaturation by 0.5% (w/v) SDS than the wild-type NC1 trimer. This indicates that the conformation of the NC1 domain of human collagen X is altered by the Y598D substitution, such that the resulting trimers are destabilized. In support of this, the Y598D-engineered human NC1 domain expressed in Escherichia coli showed a dramatic reduction in stability and solubility when compared with the wild-type NC1 domain (28).

Despite the apparent wealth of relevant literature, an understanding of the full effect of SMCD mutations on the assembly and secretion of α1(X) chains is lacking. To overcome the limitations of transient transfection, where expression levels are difficult to ascertain, or synthesis of mutant α1(X) chains in vitro, in the absence of the complete secretory pathway quality control, we generated stably transfected cells expressing wild-type and SMCD mutant α1(X) chains. Although previous transfection studies have confirmed degradation of the two specific SMCD mutations that prevent cleavage of the signal peptide (17), our studies are the first to demonstrate the effect of two NC1 domain mutations, Y598D and NC1A10, on the secretion of human α1(X) chains in a cellular context. Here we have shown that, although normal chains are secreted, mutant chains are selectively retained and are degraded via both proteasomal and lysosomal/endosomal pathways, despite evidently high expression levels at the mRNA level.

Although both SMCD mutations severely compromised α1(X) chain assembly and folding, we observed small amounts of triple-helical Y598D and NC1A10 chains in both semi-permeabilized cells and transfected cells. However, only protease-resistant material formed by wild-type α1(X) chains could be detected in the media fraction. Studies on the secretion of procollagen from fibroblasts indicate that the rate-limiting step in secretion is triple-helix formation (29). Our data are therefore the first to demonstrate that mutant collagen chains with correctly aligned helices can be selectively retained. One interpretation of this result is that correct folding and assembly of the other domains, most likely the NC1 domain, is critical for secretion and that mutant chains are recognized by a highly selective quality control mechanism in intact cells (30, 31).

The specific interaction between mutant α1(X) chains and PDI identifies one endoplasmic reticulum protein that could function as part of such a quality control mechanism. Based on cross-linking studies using bovine α1(X) chains, it has been shown that PDI does not differentiate between the conformation of normal and mutant trimers (16). As discussed above, it appears that SMCD mutations affect the assembly of human NC1 domains more severely than bovine NC1 domains. It has been demonstrated that mis-folding of the human Y598D NC1 domain expressed in E. coli results in specific association with the bacterial chaperone GroEL (28). We did not detect any interaction with BiP, the mammalian homologue of GroEL, which is consistent with published results (16). Our results suggest that the SMCD mutant α1(X) chains adopt a conformation recognized by PDI, resulting in retention within the endoplasmic reticulum. PDI is also intimately linked with the β subunit of P4-H. This enzyme function as a catalyst of proline hydroxylation and as a chaperone, which mediates retention of incompletely folded procollagen chains within the endoplasmic reticulum (24, 26, 32). Our data show that triple-helix formation by NC1 mutants is inefficient compared with the wild-type chains. Therefore, further investigation will determine whether PDI acts independently or as a subunit of P4-H during the retention of mutant α1(X) chains.

The main objective of this study was to assess the extent to which co-assembly and secretion of normal-mutant α1(X) heterotrimers contributes to the SMCD disease mechanism. Critical analysis of the data published by this group and others argues that the SMCD phenotype cannot be explained comprehensively by a single disease mechanism. In two individuals, the introduction of premature termination codons within the mutant mRNA results in functional haploinsufficiency due to nonsense-mediated decay of the mutant allele (15). In another report, mRNA encoding the missense mutant Y598D was detected within the growth plate cartilage of one SMCD patient carrying this allele (33). It is not clear whether the NC1A10 mutant allele is stable in vivo. However, the resulting mRNA is truncated by only 27 bp and therefore may escape mutant mRNA surveillance and degradation. Although it was initially reported that several SMCD mutations abolished NC1 trimer formation, it has been demonstrated that, in vitro systems, wild-type and SMCD mutant α1(X) chains are capable of heterotrimer formation, supporting a dominant negative disease mechanism. Such a mechanism where mutant chains are “fully” included in α1(X) trimer formation has two potential outcomes. One is that heterotrimers incorporating one or more mutant chains are unstable and are targeted for intracellular degradation, reducing the secretion of functional collagen X chains by nearly 90%. The other possibility is that SMCD mutations do not destabilize assembled trimers, resulting in secretion of mutant homotrimers and heterotrimers that interfere with collagen X assembly and interactions within the cartilage extracellular matrix. In the present study we demonstrated that, in intact cells, mutant chains are largely excluded from co-assembly with normal α1(X) chains and that only a very minor fraction of Y598D chains had formed stable heterotrimers in the endoplasmic reticulum. Furthermore, Y598D and NC1A10 chains were secreted in only trace amounts, indicating that α1(X) chains harboring these mutations are retained and degraded within the cell. It is uncertain the extent to which such a minor population of normal-mutant heterotrimers would contribute to matrix dysfunction within the developing cartilage of SMCD patients. We also show that, compared with cells expressing wild-type α1(X) chains alone, secretion of normal chains from co-transfected cells is not dramatically reduced.

In conclusion, our data strongly indicate that, in stably transfected cells, mutant α1(X) chains harboring the Y598D and NC1A10 SMCD mutations are excluded from trimer formation. Therefore, we propose that, in contrast to the dominant negative model described above, their expression principally results in functional haploinsufficiency. Importantly, further studies are required to examine the effects of other SMCD mutations within stably transfected cells. Mapping of SMCD mutations onto the NC1 trimer modeled on the crystal structure of ACRP30 (20) raises the possibility that other mutations can be tolerated within stable homo- and heterotrimers. Studies on how these mutations affect supramolecular assembly and interactions with other cartilage matrix components will provide further insight into the molecular pathology of SMCD and consequently the role of collagen X in growth plate cartilage function.

REFERENCES
1. Schmid, T. M., and Linsenmayer, T. F. (1985) J. Cell Biol. 100, 598–605
2. Reichenberger, E., Aigner, T., von der Mark, K., Stoss, H., and Bertling, W. (1991) Dev. Biol. 148, 562–572
3. Grant, W. T., Wang, G. J., and Biilian, G. (1997) J. Biol. Chem. 272, 9844–9849
4. Kwan, K. M., Pang, M. K., Zhou, S., Cowan, S. K., Kong, R. Y., Pfordte, T., Olsen, B. R., Silence, D. O., Tam, P. P., and Cheah, K. S. (1997) J. Cell Biol. 136, 459–471
5. Kwan, A. P., Cummings, C. E., Chapman, J. A., and Grant, M. E. (1991) J. Cell Biol. 114, 597–604
6. Poole, A. R., and Pidoux, I. (1989) J. Cell Biol. 109, 2547–2554
7. Schmid, T. M., and Linsenmayer, T. F. (1989) Connect. Tissue Res. 20, 215–222
8. Warman, M. L., Abbott, M., Apte, S. S., Hefferon, T., McIntosh, I., Cohn, D. H., Hecht, J. T., Olsen, B. R., and Francomeo, C. A. (1993) Nat. Genet. 3, 79–82
Selective Retention and Degradation of SMCD Mutant Collagen X Chains

9. Wallis, G. A., Rash, B., Sweetman, W. A., Thomas, J. T., Super, M., Evans, G., Grant, M. E., and Boot-Handford, R. P. (1994) *Am. J. Hum. Genet.* **54**, 169–178
10. Wallis, G. A., Rash, B., Sykes, B., Bonaventure, J., Maroteaux, P., Zabel, B., Wyne-Davies, R., Grant, M. E., and Boot-Handford, R. P. (1996) *J. Med. Genet.* **33**, 450–457
11. Ikegawa, S., Nakamura, K., Nagano, A., Haga, N., and Nakamura, Y. (1997) *Hum. Mutat.* **9**, 131–135
12. Brass, A., Kadler, K. E., Thomas, J. T., Grant, M. E., and Boot-Handford, R. P. (1992) *FEBS Lett.* **303**, 126–128
13. Chan, D., Weng, Y. M., and Bateman, J. F. (1999) *J. Biol. Chem.* **274**, 13091–13097
14. Chan, D., Cole, W. G., Rogers, J. G., and Bateman, J. F. (1995) *J. Biol. Chem.* **270**, 4558–4562
15. Chan, D., Weng, Y. M., Graham, H. K., Sillence, D. O., and Bateman, J. F. (1996) *J. Clin. Invest.* **101**, 1490–1499
16. McLaughlin, S. H., Conn, S. N., and Bulleid, N. J. (1999) *J. Biol. Chem.* **274**, 7570–7575
17. Chan, D., Weng, Y. M., Hocking, A. M., Golub, S., McQuillan, D. J., and Bateman, J. F. (1996) *J. Biol. Chem.* **271**, 13566–13572
18. Dharmavaram, R. M., Elberson, M. A., Peng, M., Kirson, L. A., Kelley, T. E., and Jimenez, S. A. (1994) *Hum. Mol. Genet.* **3**, 507–509
19. Wilson, R. R., and Bulleid, N. J. (2000) *Methods Mol. Biol.* **139**, 1–9
20. Marks, D. S., Gregory, C. A., Wallis, G. A., Brass, A., Kadler, K. E., and Boot-Handford, R. P. (1999) *J. Biol. Chem.* **274**, 3632–3641
21. Gurevich, V. V., Pokrovskaya, I. D., Obukhova, T. A., and Zozulya, S. A. (1991) *Anal. Biochem.* **195**, 207–213
22. Wilson, R., Allen, A. J., Oliver, J., Brookman, J. L., High, S., and Bulleid, N. J. (1995) *Biochem. J.* **307**, 679–687
23. Bengtsson, E., Aspberg, A., Heinegård, D., Sommarin, Y., and Spillmann, D. (2000) *J. Biol. Chem.* **275**, 40695–40702
24. Wilson, R., Lees, J. F., and Bulleid, N. J. (1998) *J. Biol. Chem.* **273**, 9637–9643
25. Tasab, M., Batten, M. R., and Bulleid, N. J. (2000) *EMBO J.* **19**, 2204–2211
26. Walmsley, A. R., Batten, M. R., Lad, U., and Bulleid, N. J. (1999) *J. Biol. Chem.* **274**, 14884–14892
27. McLaughlin, S. H., and Bulleid, N. J. (1998) *Biochem. J.* **331**, 793–800
28. Dublet, B., Vernet, T., and van der Rest, M. (1999) *J. Biol. Chem.* **274**, 18099–18105
29. Kao, W. W., Berg, R. A., and Prockop, D. J. (1977) *J. Biol. Chem.* **252**, 8391–8397
30. Lamande, S. R., and Bateman, J. F. (1999) *Semin. Cell Dev. Biol.* **10**, 455–464
31. Hammond, C., and Helenius, A. (1995) *Curr. Opin. Cell Biol.* **7**, 523–529
32. Chessler, S. D., and Byers, P. H. (1992) *J. Biol. Chem.* **267**, 7751–7757
33. Gregory, C. A., Zabel, B., Grant, M. E., Boot-Handford, R. P., and Wallis, G. A. (2000) *J. Med. Genet.* **37**, 627–629
34. Thomas, J. T., Cresswell, C. J., Rash, B., Nicolai, H., Jones, T., Solomon, E., Grant, M. E., and Boot-Handford, R. P. (1991) *Biochem. J.* **266**, 617–623
35. Reichenberger, E., Beier, F., LaValle, P., Olsen, B. R., von der Mark, K., and Bertling, W. M. (1992) *FEBS Lett.* **311**, 305–310