ARTICLE

Homozygosity for a Missense Mutation in *SERPINH1*, which Encodes the Collagen Chaperone Protein HSP47, Results in Severe Recessive Osteogenesis Imperfecta

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Osteogenesis imperfecta (OI) is characterized by bone fragility and fractures that may be accompanied by bone deformity, dentinogenesis imperfecta, short stature, and shortened life span. About 90% of individuals with OI have dominant mutations in the type I collagen genes *COL1A1* and *COL1A2*. Recessive forms of OI resulting from mutations in collagen-modifying enzymes and chaperones *CRTAP*, *LEPRE1*, *PPIB*, and *FKBP10* have recently been identified. We have identified an autosomal-recessive missense mutation (c.233T>C, p.Leu78Pro) in *SERPINH1*, which encodes the collagen chaperone-like protein HSP47, that leads to a severe OI phenotype. The mutation results in degradation of the endoplasmic reticulum resident HSP47 via the proteasome. Type I procollagen accumulates in the Golgi of fibroblasts from the affected individual and a population of the secreted type I procollagen is protease sensitive. These findings suggest that HSP47 monitors the integrity of the triple helix of type I procollagen at the ER/*cis*-Golgi boundary and, when absent, the rate of transit from the ER to the Golgi is increased and helical structure is compromised. The normal 3-hydroxylation of the prolyl residue at position 986 of the triple helical domain of proα1(I) chains places the role of HSP47 downstream from the CRTAP/P3H1/CyPB complex that is involved in prolyl 3-hydroxylation. Identification of this mutation in *SERPINH1* gives further insight into critical steps of the collagen biosynthetic pathway and the molecular pathogenesis of OI.

Introduction

Osteogenesis imperfecta (OI [MIM 166200, 166210, 259420, and 166220]) or "brittle bone disease" ranges in severity from lethal in the newborn period to only mild increase in fracture frequency.[1,2] OI is most often the result of dominant mutations in the type I collagen genes *COL1A1* (MIM 120150) and *COL1A2* (MIM 120160). A wide array of mutations that affect the type I collagen triple helix (most of which result in substitutions of glycine residues in the uninterrupted Gly-X-Y triplet repeat of the 1014 residue triple helical domain) and globular carboxy-terminal region (C-propeptide) have been identified, and attempts have been made to draw correlation between the site of mutation and severity of the phenotype.[3,4] In spite of the large collection of mutations, the only clear genotype-phenotype relationship is that premature termination codons in the *COL1A1* gene result in mild OI type I whereas almost all mutations that result in collagen structural abnormalities produce more severe phenotypes. Although there is more to be understood about type I collagen and the pathogenesis of OI, the link between mutation and phenotype may lie not in the more prevalent dominant forms of OI but in the recently recognized recessive mutations that target key points in the downstream collagen biosynthesis pathway.

In a fraction of individuals, OI is inherited in an autosomal-recessive manner and mutations in genes that encode four endoplasmic reticulum (ER) proteins that lead to OI were identified recently[5–10] (U. Schwarze et al., 2009, Am. Soc. Hum. Gen., abstract; Y. Alanay et al., 2009, Am. Soc. Hum. Gen., abstract). The triple helical domain, which is characterized by a repeating Gly-X-Y triplet, of the constituent chains of type I procollagen undergoes a series of posttranslational modifications in the ER. These include hydroxylation of Y-position prolyl residues at the 4-position of the ring, hydroxylation of some Y-position lysyl residues, and glycosylation of hydroxylated lysyl residues. A prolyl residue at position 986 of the proα1(I) triple helix is hydroxylated at the 3-position of the ring by a complex comprising cartilage-associated protein (CRTAP [MIM 605497]), prolyl 3-hydroxylase (P3H1 [MIM 610339]), and cyclophilin B (CyPB [MIM 123841]), encoded by the *CRTAP*, *LEPRE1*, and *PPIB* genes, respectively. Recessive mutations leading to OI have been identified in all three members of this complex (U. Schwarze et al., 2009, Am. Soc. Hum. Gen., abstract).[5–9] Mutations in *COL1A1*, *COL1A2*, *CRTAP, LEPRE1*, and *PPIB* usually result in overmodification (probably excess lysyl hydroxylation and glycosylation) of proα chains in the ER, presumably because of their delayed assembly into trimers.

There is a subset of individuals with OI whose cells do not overmodify collagen chains. Recessive mutations in *FKBP10* (MIM 607063), which encodes the ER-resident *cis-trans* isomerase FKBP65, were recently identified in

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several individuals whose cells did not overmodify collagens (Y. Alanay et al., 2009, Am. Soc. Hum. Gen., abstract, and unpublished results). We screened 30 individuals with OI whose cells did not produce overmodified type I collagen for mutations in *FKBP10* and *SERPINH1,* because the latter is a collagen-binding protein and functions as a chaperone in the ER. In one individual, we identified an autosomal-recessive mutation in *SERPINH1* (MIM 600943), the gene that encodes HSP47, a chaperone-like protein for collagens, and in two others we identified mutations in *FKBP10* (unpublished). A recessive mutation in *SERPINH1* leading to an OI phenotype in Dachshunds was recently identified by homozygosity mapping, but its molecular mechanism was not characterized.[11] Here we describe the human mutation in *SERPINH1* and its effect on type I procollagen production and structure.

Material and Methods

Cell Culture, Isolation of DNA and RNA, and Collagenous Protein Analysis

Human dermal fibroblasts were obtained with appropriate consent and cultured as previously described.[12] DNA and RNA were isolated with QIAGEN QIAamp DNA Mini kit and RNeasy Mini kit, respectively, per the manufacturer's instructions (QIAGEN, Valencia, CA). Complementary DNA was synthesized from RNA with Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Radiolabeling and analysis of collagenous proteins was performed as previously described.[12]

Pulse-Chase Studies

Fibroblasts from the affected individual and a control were plated at confluence (250,000 cells) in 35 mm tissue-culture dishes in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, allowed to attach overnight, and then incubated in the presence of 50 μg/ml of ascorbic acid for 18 hr prior to labeling. Confluent cells were starved of proline with serum-free medium supplemented with 50 μg/ml ascorbic acid for 1 hr, then labeled in serum-free medium with 100 μCi/ml [2,3,4,5-3H] proline (American Radiolabeled Chemicals, Inc., St. Louis, MO). After 60 min in label, cells were rinsed twice with serum-free medium with 50 μg/ml ascorbic acid, and fresh chase medium was added that contained 10 mM proline. At 0, 20, 40, 60, and 120 min, the medium was collected and the cell layer scraped into a separate tube in PBS, protease inhibitors were added to both, and the collagenous proteins were precipitated with 50% v:v ethanol and carrier collagen in the presence of 15 mM iodoacetamide added to prevent new disulfide bond formation. Precipitated proteins were suspended in SDS-sample buffer and separated on 5% SDS-PAGE under nonreducing condition. The gels were fixed, treated with Enhance (Perkin Elmer, Waltham, MA), and dried. Radiolabeled bands were visualized by autoradiography. Band density was quantitated with the ImageJ software.

Subject Selection

Because of its role as a collagen chaperone and collagen binding protein,[13] *SERPINH1* was sequenced in DNA from 30 individuals whose cells made only normal type I collagen molecules via standard analytic methods.[12] Of these, 24 had a diagnosis of OI type II or OI type III, of whom 8 derived from consanguineous matings. Five had OI type III/IV based on clinical description and of these three had related parents. In one instance there was insufficient clinical information to identify the type of OI. *FKBP10* was also sequenced in all, and two individuals with OI type III had mutations identified (unpublished results).

PCR and Sequencing

SERPINH1 exons and flanking splice sites and intronic sequences were amplified from genomic DNA by PCR and sequenced (primers and conditions are available upon request). Sequences were analyzed via Mutation Surveyor V3.20 (Softgenetics).

Western Blot and Immunocytochemistry

Dermal fibroblasts were treated with 50 μg/ml of ascorbic acid overnight in DMEM with 10% fetal calf serum. For western blot, proteins were extracted by ethanol precipitation, separated by SDS-polyacrylamide gel electrophoresis on 5% or 10% gel, and transferred to nitrocellulose membrane. Membranes were probed with the following antibodies: anti-HSP47 monoclonal (SPA470, StressGen Biotechnologies, Ann Arbor, MI) (1:500) and anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA) (1:50). Peroxidase-conjugated secondary antibodies were used and detected with ECL Plus Western Blotting Detection System (GE Healthcare, Piscataway, NJ). Immunocytochemistry was performed as described (Abcam protocols) with the following antibodies: anti-Hsp47 (1:5000), anti-PDI (StressGen Biotechnologies, Ann Arbor, MI) (1:400), anti-bovine proα1(I) amino-propeptide (LF-9, 1:800),[14] anti-58K Golgi protein (Abcam, Cambridge, MA) (1:1000), and anti-KDEL (Abcam) (1:400). The anti-Hsp47 antibody was titrated with mouse *SERPINH1*$^{-/-}$ cell line KO-13, generously provided by Dr. Nagata, Kyoto University, Kyoto, Japan.[15]

Proteasome Degradation Assays

Dermal fibroblasts were treated with 50 μg/ml of ascorbic overnight and then treated with 50 μM MG-132 proteasome inhibitor (Calbiochem, La Jolla, CA) in serum-free medium with 50 μg/ml ascorbate for 0–16 hr. In additional studies, dermal fibroblasts were treated with proteasome inhibitor MG-132 for 16 hr and then with 100 μg/ml cycloheximide (CHX) and MG-132 or CHX alone for an additional 8 hr to prevent further protein synthesis. Proteins were harvested by ethanol precipitation as previously described.[12]

Prolyl 3-Hydroxylation

The extent of prolyl 3-hydroxylation in proα1(I) chains was measured as described by Morello et al.[5]

Thermostability Assay

Thermostability was determined as described by Pace et al.[16]

Protease Digestion Assay

Dermal fibroblasts were plated to confluency in 35 mm dishes and labeled with 100 μCi/ml [2,3,4,5-3H]proline in serum-free medium with 50 μg/ml ascorbic acid overnight. The medium was collected and maintained at 37°C, and secreted proteins were digested with 200 μg/ml trypsin and 500 μg/ml chymotrypsin as previously described.[15]

Cyanogen Bromide Mapping

Cleavage of collagens with cyanogen bromide and peptide mapping were performed as previously described.[12]

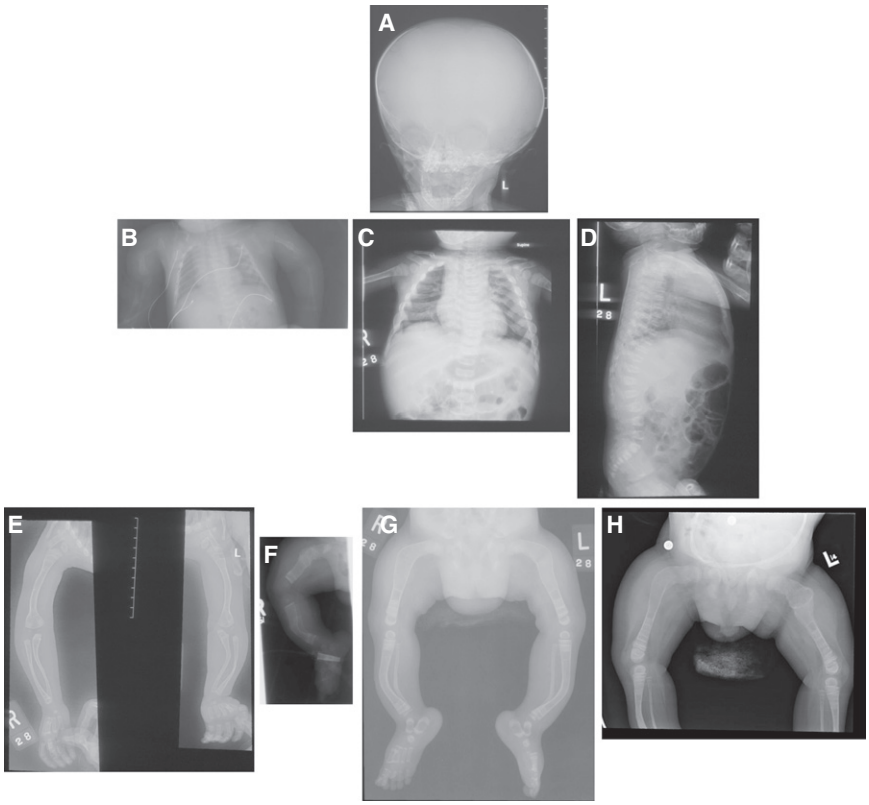


Figure 1. X-Rays
Radiographs of the skull at age 1 year (A) demonstrate diminished calvarial mineralization and enlargement of the calvarial vault. At birth, the chest (B) was small, the ribs were thin and some had healing fractures, one clavicle was bent, both humeral bones had fractures, platyspondyly was present, and overall mineralization appeared diminished. By 1 year of age (C and D), the ribs had become broad, platyspondyly was still apparent with diminished central mineralization of the vertebral bodies, and the humeral bones had a tubular shape, thin cortices, and diminished mineralization. The deformity and decreased mineralization of the bones in the arms are apparent at 1 year (E), and the minimal effect of pamidronate can be seen. At 1 month (F), 1 year (G), and 28 months (H), there is rhizomelic shortening and limited modeling of the long bones with very thin cortices. The lines of increased mineralization reflect the successive pamidronate infusions.

Results

Clinical Report

The index case was the first child born to a clinically normal consanguineous Saudi Arabian couple and was the only affected member in the extended family (Figures 1 and 2A). At about 28 weeks gestation, fetal ultrasound, performed because the previous scans at an outside hospital were not available, identified short bowed femora. A repeat scan at 36 weeks and 5 days again demonstrated short femora, low echogenicity of the calvarium, and a relatively small chest. No internal organ abnormalities were noted. There were no maternal medications taken and no intercurrent illnesses during pregnancy.

The boy was born by elective cesarean section at 38 weeks gestation because of breech presentation; the Apgar scores were 7 and 9 at 1 and 5 min, respectively. Birth weight was 2.43 kg (10^{th}–25^{th} centile) and head circumference was 33.5 cm (25^{th} centile). Birth length was not recorded.

At birth he was noted to have a triangular face, relative macrocephaly, bitemporal narrowing, blue sclerae, micrognathia, and relatively short limbs with bowing at the thighs. Radiographs of the chest at birth showed thin ribs with healing fractures, a fracture of the right humerus, a healing fracture of the left humerus, and platyspondyly (Figure 1B). His fractures were managed by splinting and analgesia. He had respiratory distress that required ventilation for 1 day followed by nasal continuous positive airway pressure for 2 weeks. At 1 week of age, he was noticed to have a few localized skin blisters and bullae. A skin biopsy was done to rule out epidermolysis bullosa simplex (EB simplex [MIM 131900]); the histopathology report was not consistent with EB. The bullae resolved shortly after birth, no new bullae formed, and no additional studies were completed.

At 3 weeks of age, pyloric stenosis was corrected by pyloromyotomy and antigastroesophageal reflux medications were begun; he also had a bilateral inguinal hernia repair. A skeletal survey at the age of 1 month was consistent with the diagnosis of osteogenesis imperfecta. There were multiple bone deformities and fractures that involved the upper and lower extremities (Figure 1F) and ribs and generalized osteopenia. He was treated with pamidronate infusion at the age of 1 month with the supplement of vitamin D3 (800 units) and elemental calcium for a total of 2 weeks postinfusion. The pamidronate was initially given by infusion every 3 to 4 months because of shortage of hospital beds; this was changed to every 2 months at the age of 2 years. He was followed with regular renal ultrasounds, and at the age of 1 year bilateral renal stones were noticed with left pelviureteric junction obstruction that ultimately required left nephrectomy because of hydronephrosis and loss of renal function. He developed bilateral subdural hematomas secondary to trauma at 1 year of age that was ultimately managed by bilateral shunt. He had chronic lung disease of unclear etiology and was admitted to the hospital several times with pulmonary infections and wheezing; from the age of 1.5 years he required 0.5–1 L of continuous oxygen by nasal cannula to maintain adequate oxygenation.

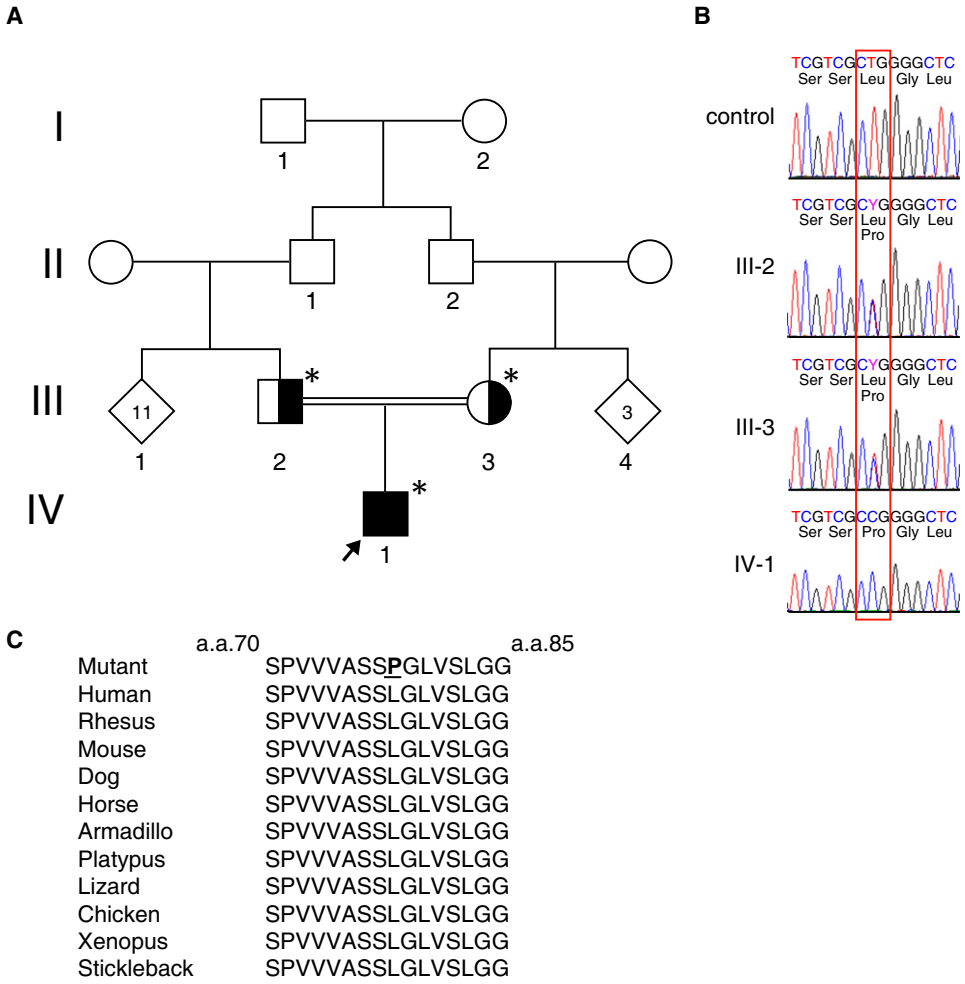


Figure 2. Identification of a Homozygous Mutation in *SERPINH1*
(A) Family pedigree with consanguineous heterozygous parents (III-2 and III-3) and affected proband (IV-1, black arrow). Asterisks indicate individuals studied.
(B) Homozygous c.233T>C, p.Leu78Pro mutation in exon 1 of *SERPINH1* in proband, IV-1 (red box). Parents (III-2 and III-3) were heterozygous at this site.
(C) The completely conserved 16 amino acid sequence in HSP47 that contains the leucine-to-proline substitution.

The skeletal survey at the age of 1 year showed decreased bone density, a narrow chest, broad ribs, thoracic scoliosis, platyspondyly, deformity of all bones of the arms, a fracture in the proximal shaft of the left femur, bilateral bowing of tibiae and fibulae, and osteopenia (Figures 1A, 1C–1E, and 1G). In the last skeletal survey at the age of 2 years and 4 months, there was thoracic scoliosis convex to the left, generalized osteopenia, multiple healed fractures on the left ribs, a fracture in the proximal shaft of the left humerus, right femoral fractures of various ages, and bilateral bowing of the femora, tibiae, and fibulae (Figure 1H). Bone mineral density of the lumber spine (L2–L4) was 0.101 g/cm^2, 0.246 gm/cm^2, and 0.210 g/cm^2 at the ages of 4 months, 1 year, and 2 years, respectively.

At the last examination at age 3 years and 5 months, he weighed 7.81 kg (<5th centile), was 60 cm (<5th centile) in length, and his head circumference was 49 cm (25th centile). He had relative macrocephaly, an open anterior fontanel, high and prominent forehead, midface hypoplasia with shallow orbits, and blue sclerae. He had small opalescent teeth consistent with dentinogenesis imperfecta. His chest was asymmetrical with bulging on the left side and thoracic scoliosis convex to the left. He had short upper and lower limbs, bowed thighs, and valgus angulation at the knee with generalized joint laxity. Neurologically he was alert, smiling, and cognitively intact according to his mother. He rolled over with difficulty and was unable to sit because of head lag and generalized hypotonia. He was able to lift his legs to his head level when lying on his back, and could hold, transfer, and give objects. He had a high pitched voice, communicated with single words, and had a vocabulary of around 30 words. His abdomen, cardiovascular system, and skin were unremarkable.

At age 3 years and 6 months he had sudden unexplained respiratory distress at home. Upon arrival to the hospital, he was cyanosed and needed resuscitation and intubation but died soon afterwards. No autopsy was performed.

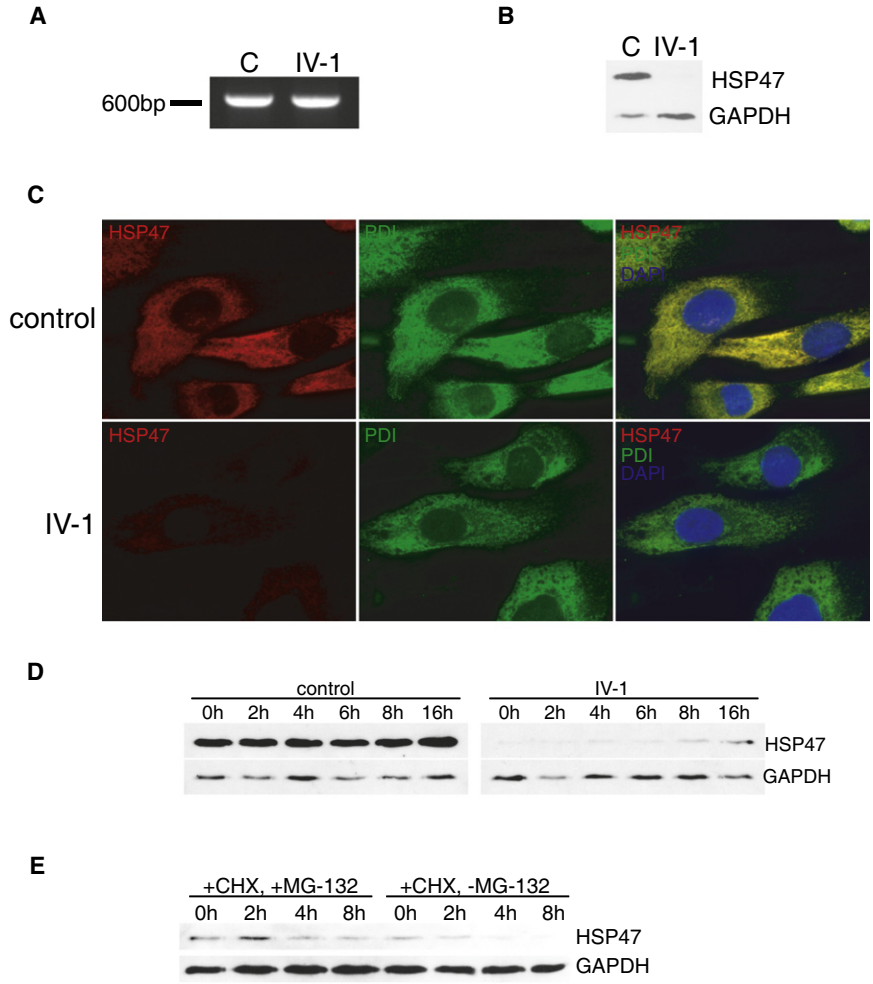


Figure 3. Mutation of *SERPINH1* Generates an Unstable Protein

(A) PCR amplification of cDNA (35 cycles for each) from control (C) and from patient (IV-1) showed that the mRNA for *SERPINH1* was stable.

(B) Western blot analysis indicated that HSP47 was significantly decreased in patient cells (IV-1).

(C) Immunocytochemistry with antibodies to HSP47 and the ER marker protein disulfide isomerase (PDI) showed a normal ER distribution of HSP47 in control cells and a severe reduction in HSP47 in patient cells (IV-1). The merged image (right column) confirms the absence of staining for HSP47.

(D) Western blot showed partial rescue of HSP47 beginning at ~8 hr of treatment with proteasome inhibitor MG-132.

(E) HSP47 was stabilized after treatment of cells with cycloheximide (CHX) and MG-132 but in the absence of MG-132, even with continuous presence of CHX, previously stabilized HSP47 was destroyed.

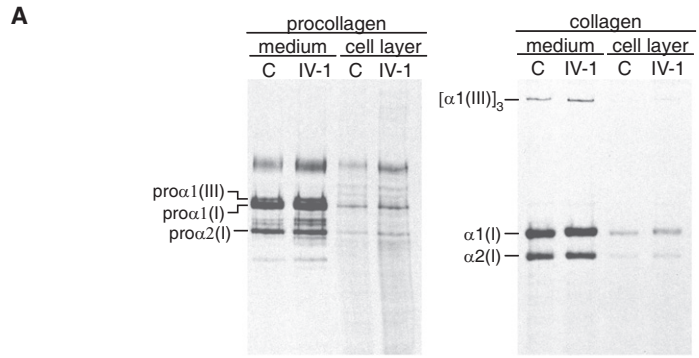
Mutation of *SERPINH1* Results in Severe Deforming OI

When analyses of the *COL1A1*, *COL1A2*, *CRTAP,* and *LEPRE1* genes were normal and collagens synthesized by cultured fibroblasts had normal modification, we sequenced *FKBP10* and *SERPINH1* and identified a homozygous missense mutation in *SERPINH1* (c.233T>C, p.Leu78Pro) (Figures 2B and 2C). The phenotypically normal parents of the proband were heterozygous for the mutation (Figure 2B). The mutant *SERPINH1* mRNA transcript was stable in dermal fibroblasts (Figure 3A), but western blot and immunocytochemical analysis showed that the protein product HSP47 was significantly decreased in patient cells (Figures 3B and 3C). This was confirmed by western blot with a second antibody that recognized an epitope near the carboxy-terminal end of HSP47 (data not shown).

To determine the mechanism of protein degradation, we treated fibroblasts with proteasome inhibitor MG-132 for 0–16 hr and found that the mutant protein was partially recovered beginning at 8 hr (Figure 3D). To confirm that mutant protein stabilization resulted from inhibition of the proteasome and not abnormal gene activation, fibroblasts were treated with MG-132 for 16 hr to allow mutant protein to accumulate and then with MG-132 with cycloheximide (CHX) or CHX alone to arrest translation for an additional 2–8 hr. Cells treated with both CHX and MG-132 maintained steady levels of HSP47. Cells treated only with CHX showed reduction of HSP47 after 2–4 hr of CHX treatment, indicating that reactivation of the proteasome resulted in degradation of accumulated mutant HSP47 (Figure 3E).

HSP47 Is Required for Normal Type I Procollagen Localization but Not for Procollagen Chain Modification in the ER

HSP47 is known to interact with type I procollagen although the nature of this interaction is still not completely understood.[17] Mutations in other type I collagen chaperones and modifying enzymes such as *CRTAP, LEPRE1*, and *PPIB* usually lead to overmodification of collagen chains and decreased 3-hydroxylation of the prolyl residue at position 986 of the triple helical domain of proα1(I) chains.[5–9] Analysis of collagen synthesis by patient cells showed that the amount of type I procollagen secreted and accumulated in the medium after a 16 hr period was similar to that in controls. There was no increase in intracellular storage, and posttranslational modification of type I procollagen chains was normal as evidenced by no increase in band width of proα1(I) and proα2(I) chains (Figure 4A). Although the amount of type I procollagen secreted was similar to that in control cells (Figure 4A) over a 16 hr period, the rate of secretion of type I procollagen trimers into the medium measured during pulse-chase



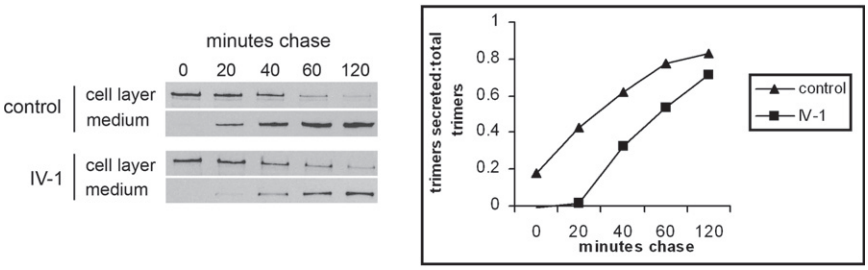


Figure 4. Type I Procollagen Synthesis and Secretion

(A) Labeling of cells with [^{3}H]-proline for 16 hr showed normal synthesis and secretion of type I procollagen into the culture medium and normal electrophoretic mobilities of the constituent chains. The chains of the procollagens were separated under reducing conditions. The collagens represent the procollagens after digestion with pepsin and electrophoresis under nonreducing conditions.

(B) Cells were labeled for 1 hr with [^{3}H]-proline then chased for up to 2 hr with unlabeled proline. The secretion of the trimers of type I procollagen showed a delay in the rate of secretion in the patient cells, quantitated in the graph to the right.

studies over a 2 hr period was delayed by a short time when compared to control (Figure 4B).

Tandem mass spectrometry analysis of secreted procollagens showed that 3-hydroxylation of the prolyl residue at position 986 of the proα1(I) triple helical domain was similar in samples from patient and control cells (Figure S1 available online), suggesting that HSP47 function is independent of the CRTAP/P3H1/CyPB complex responsible for 3-hydroxylation.

The chains of type I procollagen are synthesized, hydroxylated, gycoslated, assembled, and folded into triple helices in the ER. Trimeric type I procollagen molecules are then transported into the Golgi where oligosaccharide modifications occur. In control cells, type I procollagen was detected in both ER and Golgi (Figure 5) by immunofluorescence. In patient cells, type I procollagen was less abundant in the ER and accumulated in the Golgi (Figure 5).

HSP47 Is Important for Proper Collagen Triple Helix Stability

Posttranslational modification of the triple helix was not affected in patient cells. To determine whether loss of HSP47 changed the conformation of the type I procollagen triple helix, we examined the protease sensitivity of secreted procollagen. The triple helical domain of type I procollagen secreted by control cells is protease resistant at 37°C but the chains are degraded very rapidly when denatured at 50°C prior to protease treatment (Figure 6A). In contrast, type I procollagen secreted from patient cells was cleaved asymmetrically within the triple helical domain when digested for 1 min at 37°C with a combination of trypsin and chymotrypsin (Figure 6A). These fragments were completely lost after 5 min. These findings indicate that HSP47 is a critical monitor of the final integrity of the triple helix. Analysis of the type I collagen cleavage products by cyanogen bromide peptide mapping indicated that the major fragments seen were the products of cleavage near the collagenase site (residues 775–776 in the triple helical domains of each chain) (Figure 6B). Type III collagen secreted from patient cells was also more protease sensitive, consistent with a role for HSP47 in monitoring helix formation of multiple collagen types.

When secreted procollagen molecules were harvested by precipitation at 4°C to promote stabilization of the triple helix, the thermostability was increased by 0.5°C (Figure 6C) in cells from the patient. This suggests that at 37°C, some type I procollagen molecules produced by the HSP47-defective cells were abnormally folded and accessible to proteases, but that if the procollagen trimers were cooled prior to thermal denaturation, they formed a stable arrangement that required a slightly higher temperature to denature than molecules from control cells.

Discussion

We identified a homozygous missense mutation in *SERPINH1* (c.233T>C, p.Leu78Pro), the gene that encodes HSP47, in an individual with a severe deforming form of OI that clinically fits into the OI type III classification. The mutation resulted in degradation of virtually all the abnormal HSP47 via the proteasome. Mutation of *SERPINH1* did not affect posttranslational modification of type I procollagen. It appeared to accelerate transit of type I procollagen from the ER to the Golgi but slightly slowed the overall transit time from inside the cell to the

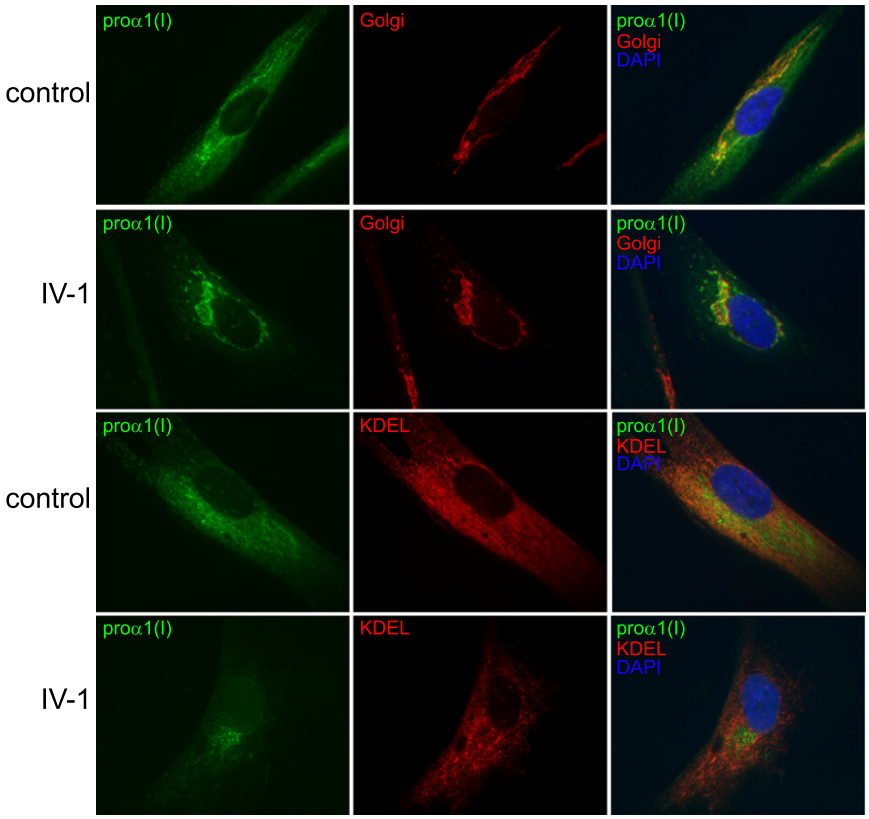


Figure 5. Type I Collagen Accumulates in the Golgi of HSP47 Mutant Cells
Immunocytochemistry with antibodies against the N-propeptide of proα1(I), Golgi marker 58K Golgi protein, and ER marker KDEL showed localization of type I procollagen to both Golgi and ER in control cells. In patient cells (IV-1 rows), type I procollagen was diminished in the ER and accentuated in the Golgi.

extracellular environment. The overall rate of type I procollagen production was, nonetheless, effectively normal, but the triple helical conformation of secreted type I collagen was altered so that secreted type I collagen was protease sensitive at one or more specific sites.

SERPINH1, a member of the serine proteinase inhibitor gene family, encodes HSP47, which was initially identified as a gelatin binding protein,[18] named coligin. It is an ER-resident protein and has an RDEL carboxy-terminal sequence that provides the ER anchor. A mouse knockout of *SERPINH1* resulted in embryonic lethality and had pleiotropic effects on collagen-containing tissues.[15] The mature processed form of type I procollagen was severely reduced in whole tissue, and type I procollagen secreted by a *Serpinh1*$^{-/-}$ mouse fibroblast cell line was protease sensitive at 37°C. In their studies, type I procollagen accumulated as aggregates in the ER and secretion was delayed.[19] Cleavage of the proα1(I) N-propeptide was also deficient in *Serpinh1*$^{-/-}$ fibroblasts, consistent with localized structural alterations of the substrate procollagen sequences. Although HSP47 was severely reduced with the human missense mutation Leu78Pro, a small amount of protein could still be detected upon longer exposure of western blot (data not shown). If the remaining protein is functional, this may account for the less severe phenotype and differences in effects, such as type I procollagen localization, in this individual. A mutation in Dachshunds that also converted a leucine residue to a proline residue (Leu326Pro), but near the carboxy-terminal end of HSP47, resulted in a viable yet severe OI phenotype.[11]

Individual proα chains are translocated into the rough ER where they undergo cotranslational modification (prolyl and lysyl hydroxylation, hydroxylysyl glycosylation, and addition of oligosaccharides). Sequences in the carboxy-terminal propeptides guide type-specific association of individual proα chains and initiate propagation of the triple helix. Trimers are trafficked to the Golgi where the glycosylated sites in the carboxy-terminal propeptide are further processed, and the trimers are packaged for secretion. Mutations in *COL1A1* and *COL1A2* that alter the protein sequence, and those in *CRTAP*, *LEPRE1*, and *PPIB*, each usually result in overmodification of proα chains of type I procollagen and retention of the trimeric molecules in the ER, suggesting that mutations in these genes affect early stages of the collagen biosynthesis, assembly, and transport pathway. Mutation of *SERPINH1* and *FKBP10* (unpublished results) do not result in overmodified collagen chains, indicating that the products of these genes probably interact with type I procollagen molecules at later stages of biosynthesis.

Although HSP47 can interact with individual chains, it appears that the preferred substrate is the triple helical domain of intact procollagen trimers.[20,21] The missense mutation we identified in *SERPINH1* appears to have several effects on type I procollagen production that we could measure: rapid exit of type I procollagen trimers from the ER to the Golgi, slightly delayed overall transit time from the ER to the extracellular space, and failure to mature all type I procollagen trimers so that they have increased protease sensitivity. These findings suggest that HSP47 may monitor the fidelity of triple helical structure at the ER/*cis*-Golgi boundary. How these changes result in OI remains unclear, but changes in triple helical structure could affect fibril assembly in the extracellular matrix and lead to irregular mineralization and an OI phenotype.

It has become clear that OI phenotypes can result from mutations in type I collagen genes and in genes that encode proteins that modify the nascent chains, monitor the secretory pathway, and assess triple helix formation of type I procollagen. With the exception of the broad

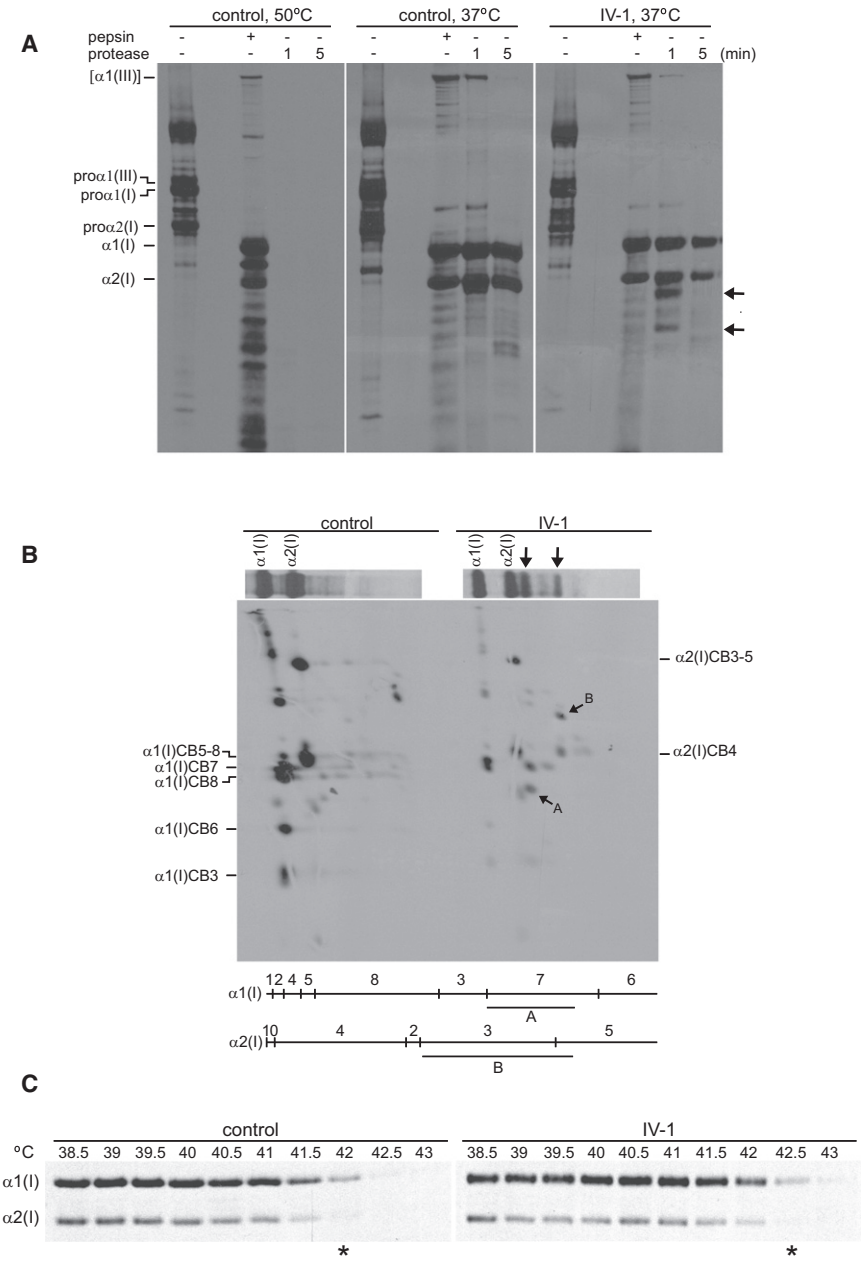


Figure 6. Mutation of HSP47 Affects Protease Sensitivity and Thermostability of the Type I Collagen Triple Helix

(A) Proteins in medium from cultured dermal fibroblasts labeled overnight with [^{3}H]-proline were digested for 1 or 5 min with a combination of trypsin and chymotrypsin at 37°C without prior cooling of the sample. Pepsin-treated samples were ethanol precipitated at 4°C and then digested for 2 hr at 16°C. Procollagens secreted into the culture medium by control cells and then pretreated at 50°C for 15 min showed complete degradation after treatment with trypsin/chymotrypsin while procollagens from medium left at 37°C had protease-resistant triple helical domains. A subset of type I procollagen molecules secreted from patient cells were cleaved asymmetrically at 37°C (the black arrows indicate fragments seen in affected cells and either not seen or in low abundance in the control cells).

(B) Characterization by cyanogen bromide peptide mapping of the cleavage products of type I procollagen after trypsin/chymotrypsin treatment. The gel lanes from runs equivalent to those in (A) were excised and the bands cleaved with cyanogen bromide. The larger of the new bands, which migrated just below α2(I), was derived from α1(I). The CB7 fragment is missing and replaced by fragment A. The size of the fragment indicates that the parent peptide had been cleaved in CB7 near the mammalian collagenase cleavage site (775–776 in the triple helical domain). The smaller fragment in the parent gel was derived from α2(I). The CB3-5 fragment was shortened, fragment B, and the estimated size indicated that it had been cleaved in the region of the collagenase cleavage site.

(C) Thermal stability of type I collagen molecules. Cells were incubated overnight with [^{3}H]-proline in the presence of sodium ascorbate (50 μg/ml). The secreted proteins were collected by precipitation with ethanol and then reheated to the temperatures shown at which point they were treated with trypsin/chymotrypsin for 2 min. The thermostability of type I collagen was increased in patient cells by about 0.5°C (*).

distinction between mutations that affect the amount of type I procollagen produced (OI type I) and those that result in production of abnormal molecules (other forms of OI), the relationship between genotype and phenotype has been difficult to discern. Although ascertainment bias cannot yet be excluded, it is striking that mutations in the genes that encode modifying and monitoring proteins cluster in the more severely affected individuals. The substrate for all forms of OI appears to be type I collagen. Mutations in the two genes that encode those proteins, *COL1A1* and *COL1A2*, suggest that monitoring triple helix integrity is critical to the "prevention" of OI. Although neither prolyl 4-hydroxylase nor peptidyl-disulfide isomerase (two components of the same protein) has yet been implicated, they remain formal candidates for mutations that result in OI because of their role in helix stabilization. Mutations in the *CRTAP, LEPRE1,* and *PPIB* complex, like mutations that alter the sequences of the triple helical domain of the proα chains, usually result in overmodified procollagens. The common mechanism to these is not yet clear but may be the consequence of failure of the CyPB protein, encoded by *PPIB*, to create the correct substrate for the prolyl 4-hydroxylase. Because mutations in *SERPINH1* and *FKBP10* do not result in overmodified chains, they appear to act downstream from the former mutations. Finally, the last of the known mutations that

result in an OI phenotype occur in *PLOD2* and result in autosomal-recessive Bruck syndrome, which is characterized by short stature, fractures, and contractures.[22] Mutations in *PLOD2* alter hydroxylation of lysyl residues in the telopeptide domains of type I procollagen chains. Although this is a modification thought to occur on chains prior to trimer assembly, its absence does not interfere with helix formation and thus appears to act further downstream, perhaps at the point of matrix mineralization.

Mutations at diverse sites along the type I collagen triple helix are likely to interact differently with modifying enzymes and chaperones to effect phenotypes. Even closely spaced mutations within the triple helix could interrupt binding to different chaperones or modifying proteins. This might explain the sometimes marked variation in clinical outcome of these sequence alterations. We noted that protease digestion of type I procollagen synthesized by HSP47-deficient cells generated a distinctive ladder of cleavage products (Figure 6A), with a major early cleavage near the collagenase cleavage site, which suggests that HSP47 interacts with specific sites along the collagen triple helix, consistent with the earlier identification of sites in the triple helical domain enriched in arginine as candidate sites.[21] Thus, mutations at HSP47 binding sites in type I collagen could play an additive role and affect clinical outcome more than mutations in nonbinding sites and might help to explain the marked variation of phenotypes along the chains that result from substitutions of obligate glycines by small amino acids (serine, cysteine, and alanine).[3]

Two final points need to be stressed. First, even with the identification of genes in which mutations result in recessive forms of OI, we and others have additional individuals in which rigorous search for mutations in all known causative genes has failed to identify the culprit. Thus, more genes await characterization as harbors of causative OI mutations and their identification should help to better understand the detailed molecular mechanisms of OI. Second, these findings have implications for the strategies by which individuals thought to have OI should be evaluated at the molecular genetic level.

Supplemental Data

Supplemental Data include one figure and can be found with this article online at http://www.cell.com/AJHG.

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Web Resources

The URLs for data presented herein are as follows:

ImageJ, http://rsbweb.nih.gov/ij/
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/

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