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A Point Mutation in a Type I Procollagen Gene Converts Glycine 748 of the α1 Chain to Cysteine and Destabilizes the Triple Helix in a Lethal Variant of Osteogenesis Imperfecta*

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Synthesis of type I procollagen was examined in fibroblasts from a proband with a lethal perinatal variant of osteogenesis imperfecta. After trypsin digestion of the type I procollagen, a portion of the α1(I) chains was recovered as disulfide-linked dimers. Digestion of the protein with vertebrate collagenase and mapping of cyanogen bromide peptides suggested that a new cysteine residue was present between residues 551 and 775 of the α1(I) chain. Sequencing of cloned cDNAs prepared using mRNA from the proband's fibroblasts demonstrated that some of the clones contained a single base mutation that converted the glycine codon in amino acid position 748 of the α1(I) chain to a cysteine codon.

About 80% of the type I procollagen synthesized by the proband's fibroblasts had a decreased thermal stability. The results, therefore, were consistent with the conclusion that normal pro-α1(I) chains and pro-α1(I) chains containing a cysteine residue in the α chain domain were synthesized in about equal amounts and incorporated randomly into type I procollagen. However, only about 10% of the α1(I) chains generated by trypsin digestion were disulfide-linked. Further studies demonstrated a decreased rate of secretion of type I procollagen containing the new cysteine residue and decreased processing of the protein by procollagen N-proteinase in cultures of postconfluent fibroblasts.

Both parents were phenotypically normal and their fibroblasts synthesized only normal type I procollagen. Therefore, the mutation in the proband was a sporadic one and is very likely to have caused the connective tissue fragility that produced the lethal phenotype.

Type I collagen is the most abundant protein in connective tissues, and defects in the synthesis or structure of the type I collagen or type I procollagen have been found in several heritable disorders of connective tissue. The human disorders are primarily EDS,[1] a condition characterized by loose joints, and OI, a condition characterized by brittle bones. Both EDS and OI, however, are highly heterogeneous at both the clinical and molecular levels (for reviews, see Refs. 1-4).

To date, the most common defects in type I procollagen genes found in probands with EDS or OI are mutations that cause deletions of amino acid sequences from either the pro-α1(I) or pro-α2(I) chain (5-18), and mutations that introduce a cysteine residue into the α chain domain of the pro-α1(I) chain (19-22). Detection of these two kinds of mutations is probably explained by the fact that both are readily identified by electrophoretic analysis of type I procollagen synthesized by cultured fibroblasts, whereas information about other mutations is more difficult to obtain. One proband with moderately severe OI was homozygous for a deletion of 4 bp that altered the sequence of the last 33 amino acids of the C-propeptide of the pro-α2(I) chain (23-26). Recently, a mutation was found that converts a glycine to an arginine residue in the α1(I) chain (27).

Three OI variants have been shown to have mutations that introduce a cysteine residue into the pro-α1(I) chain of type I procollagen (19-22). In one, the new cysteine was located in the cyanogen bromide peptide α1-CB8, a fragment containing amino acid residues 123-402 of the α1(I) chain[2] (19). The mutation was found in a moderately severe variant of OI that was inherited as an autosomal dominant trait. A second OI variant was shown to have a mutation that introduced a cysteine residue into α1-CB6, the cyanogen bromide peptide containing amino acid residues 823-1014 (20). Again, the mutation was found in a moderately severe variant of OI. The amino acid replaced by the cysteine residue in these two variants was not established. A third OI variant had a mutation that was a single-base substitution converting the glycine residue in amino acid position 988 of the α1(I) chain to a cysteine. This mutation was a sporadic one that apparently produced the lethal phenotype, but the role of an uncharacterized gene producing the Marfan syndrome in the same family was unclear (21, 22).

Here, we describe a point mutation in a proband with a lethal variant of OI that converts the glycine in amino acid position 748 of the α1(I) chain to cysteine.

MATERIALS AND METHODS

The Proband and His Family—The proband (JIMM-13) was a male fetus from the second pregnancy of healthy, non-consanguinous

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[1] The abbreviations used are: EDS, Ehlers-Danlos syndrome; OI, osteogenesis imperfecta; SDS, sodium dodecyl sulfate; T_m, midpoint for the helix to coil transition of collagen or procollagen; C-propeptide, COOH-terminal propeptide; N-propeptide, NH_2-terminal propeptide; bp, base pair.

[2] Amino acid positions are numbered by the standard convention in which the first glycine of the triple helical domain of an α chain is number one. The numbers for α1(I) chains can be converted to positions in the human pro-α1(I) chain by adding 156, and numbers for the α2(I) chain can be converted to the human pro-α2(I) chain by adding 68.

parents. The first child was normal. At 25 weeks of gestation, a routine ultrasound examination disclosed short and severely malformed limbs. The skull appeared to be deformed and there was decreased skeletal density. Amniocentesis was performed and chromosomal analysis revealed a normal 46, XY karyotype. Levels of α-fetoprotein were normal. The pregnancy was terminated at 27 weeks. The fetus had severe growth retardation with a weight of 600 g, a length of 27 cm, and a head circumference of 23 cm. The limbs were short and bowed, showing stigmata of intrauterine compression. The calvarium was soft with a membraneous consistency. Numerous healed or healing fractures of the ribs were present, but internal organs appeared normal. The findings were typical of OI type II (28).

The control line of skin fibroblasts (CRL 3349) was obtained from the American Type Culture Collection (Rockville, MD).

Cell Culturing and Labeling of Proteins—Skin fibroblasts from passages 6–14 were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Gibco). Confluent cultures were incubated for 4 h in fresh medium containing 1% fetal bovine serum, 50 μg/ml ascorbate, and 50 μCi/ml L-[2,3,4,5-^{3}H]proline (102 Ci/mmol, Amersham Corp.). The medium was then removed and the cell layer was washed 3 times with 2 ml of cold phosphate-buffered saline.

Proteolytic Digestions—The cell layer proteins were analyzed after digestion either with a mixture of trypsin and chymotrypsin, or with vertebrate collagenase followed by a mixture of trypsin and chymotrypsin.

For digestion with trypsin and chymotrypsin (29), the cell layer was scraped into 0.5 ml of modified Krebs II medium (30) containing 0.1% Nonidet P-40 (Sigma). The cells were mechanically agitated for 1 min and cooled on ice. An aliquot was first preincubated at appropriate temperatures for 10 min and then a one-tenth volume of a solution containing 1 mg/ml trypsin and 2.5 mg/ml chymotrypsin (Boehringer Mannheim) was added. After 2 min, the digestion was terminated by adding a one-tenth volume of 5 mg/ml soybean trypsin inhibitor (Sigma).

Samples to be treated with vertebrate collagenase (31) were dialyzed against 10 mM $CaCl_2$ in 50 mM Tris/HCl (pH 7.5 at 4 °C) immediately after mechanical agitation. Trypsin-activated vertebrate collagenase from human skin fibroblasts was added to a concentration of 12.5 μg/ml. The mixture was incubated for 15 h at room temperature and the reaction terminated by the addition of EDTA to a final concentration of 15 mM. The samples were then digested with trypsin and chymotrypsin as described previously (32).

To prepare a sample for gel electrophoresis, it was immersed in a boiling water bath for 3 min. A one-fifth volume of 5 × electrophoresis sample buffer was added. The 5 × sample buffer contained 10% SDS, 50% glycerol, and 0.012% bromphenol blue in 0.625 M Tris/HCl buffer (pH 6.8). The sample was boiled for an additional 3 min and then stored at room temperature. Samples were analyzed by polyacrylamide gel electrophoresis using the discontinuous system of Laemmli (33).

Analysis of Cyanogen Bromide Peptides—Two-dimensional mapping of CNBr peptides was performed as described by Barsh *et al.* (34). Briefly, cell layer proteins were digested with a mixture of trypsin and chymotrypsin at room temperature as described above and the resulting α chains were separated by electrophoresis in SDS on 5% polyacrylamide gels. Individual lanes were cut out of the gel and were incubated for three 10-min periods in 70% formic acid at room temperature. The solution was replaced with 50 mg/ml of CNBr in 70% formic acid and the reaction tube was flushed with nitrogen. After a 2-h incubation in the dark at room temperature, the formic acid solution was removed. The gel slice was washed with water and equilibrated with sample buffer. The gel slice was then placed on top of a 10% polyacrylamide gel and covered with diluted sample buffer with or without 10% 2-mercaptoethanol. The sample was electrophoresed in a second dimension, and the gel was processed for fluorography (35).

Synthesis and Screening of a cDNA Library—Poly(A)-enriched mRNA was isolated from cultured fibroblasts (36) and synthesis of cDNA was performed according to the method of Gubler and Hoffman (37). To increase the percentage of cDNAs containing pro-α1(I) sequences, a 21-bp oligonucleotide that was complementary to the 3′ region of the pro-α1(I) message (38) was used to prime first strand synthesis. The oligonucleotide had the sequence (anti-sense) 5′-GAGTTTACAGGAAGCAGACAG. *Eco*RI linkers were attached to the double-stranded cDNA, ligated to λ gt10 arms (Promega), and packaged using a packaging extract supplied by Promega. The bacteriophage library contained over 10^5 recombinant clones and was screened with a 625-bp *Sma*I/*Mst*II fragment isolated from Hf-404, a cloned cDNA for a part of the human pro-α1(I) chain (38). The probe was labeled by nick translation (36).

Subcloning and Sequencing—DNA was isolated from 20 positive clones. The clones were assayed for the presence of a *Bam*HI site found near the collagenase cleavage site of the α1(I) chain (see below). Three clones containing the *Bam*HI site were digested with *Eco*RI and the inserts were isolated by electrophoresis. The inserts were digested with *Bam*HI and the two fragments were subcloned into *Eco*RI/*Bam*HI sites in the filamentous bacteriophage M13mp18. The subclones were sequenced with reverse transcriptase and dideoxynucleotides (39) using GemSeq reagents (Promega).

Secretion of Procollagen—Cultured skin fibroblasts were labeled with [^{3}H]proline for 0.5–8 h. The cell layer and the media were harvested separately and analyzed by electrophoresis. Fluorograms were scanned with an Ultrascan XL laser densitometer (LKB) and the area under each peak in the medium was divided by the sum of the total areas under the corresponding peaks in the medium and the cell layer. The values were plotted along with the standard deviation for each value ($n = 4$).

Processing of Procollagen to Collagen—These experiments were performed as described previously (41). Briefly, cultures of skin fibroblasts were grown for 4 days postconfluence and fed for 2 days with medium containing ascorbate (25 μg/ml). The cells were preincubated for 1 h with medium containing 1% fetal bovine serum, ascorbate (25 μg/ml), and β-aminopropionitrile (50 μg/ml). The cultures were then labeled for 1 h with medium containing 50 μCi/ml [^{3}H]proline and chased for 0–8 h with medium without label. Samples were analyzed by electrophoresis as described above.

RESULTS

Identification of a Disulfide-linked Peptide from the Pro-α1(I)—Fibroblasts from the proband were incubated with [^{3}H]proline for 4 h, and the cell layer was digested with a mixture of trypsin and chymotrypsin (29). Analysis of the protein by electrophoresis on a 6% polyacrylamide gel in SDS indicated that most or all of α1(I) and α2(I) chains of type I collagen migrated more slowly than the controls (Fig. 1). After incubation with 0.3 mM α,α′-dipyridyl, an inhibitor of prolyl and lysyl hydroxylase, the chains co-migrated with α1(I) and α2(I) chains from control fibroblasts (not shown). Therefore, the slower migration of the α1(I) and α2(I) chains from the proband's fibroblasts is probably explained by post-translational overmodifications (see Refs. 1 and 4).

The cell layer of the proband's fibroblasts also contained a more slowly migrating band (Fig. 1). The band migrated more rapidly than trimers of pro-α chains of type I procollagen and in about the position previously reported for disulfide-linked dimers of α1(I) chains (19–22). After reduction with 2-mercaptoethanol and electrophoresis in a second dimension on a 6% polyacrylamide gel, the band co-migrated with α1(I) chains (not shown), suggesting that the band consisted of disulfide-linked α1(I) chains.

To establish more fully the nature of the putative disulfide-linked α1 chains, cell layer proteins were digested with vertebrate collagenase and then with a mixture of trypsin and chymotrypsin. As shown in Fig. 2, the disulfide-linked dimer migrated in about the position of dimers of A-fragments of α chains (see Ref. 19). There was no evidence of any disulfide-linked component of the B-fragments of the α chains. Therefore, the results suggested that some of the proband's pro-α1(I) chains contained a new cysteine residue somewhere within the first 775 amino acids of the α1(I) domain. As also noted in Fig. 2, the A-fragments of the α chains from the proband's fibroblasts migrated slightly more rapidly than control A-fragments. This increased migration of the A-fragments is probably explained by partial digestion of their COOH termini by trypsin.

To locate the cysteine residue more precisely, the putative α1(I) dimers were separated from α1(I) and α2(I) chains by polyacrylamide gel electrophoresis in SDS in one dimension.

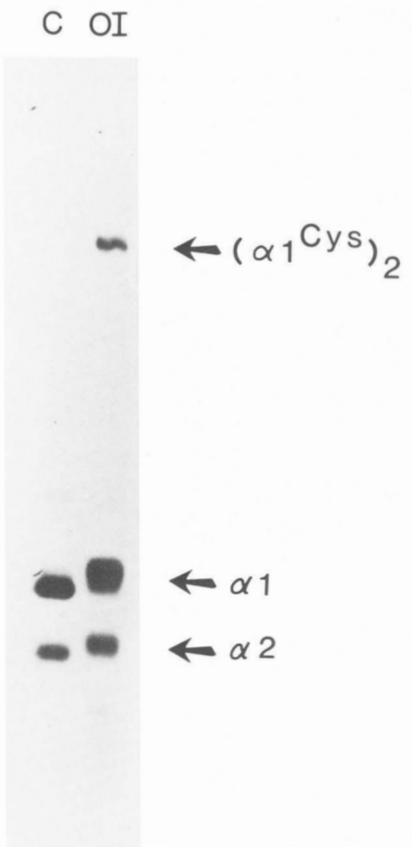

FIG. 1. **SDS-polyacrylamide gel electrophoresis of type I collagen synthesized by control (*C*) and patient's cells (*OI*).** Cells from OI and control fibroblasts were labeled for 4 h with [^{3}H] proline. The cells were scraped into Kreb's buffer containing 0.1% Nonidet P-40 and digested with trypsin and chymotrypsin. After denaturation in the presence of SDS, aliquots were electrophoresed in 5% polyacrylamide gels. Patient's cells synthesize a disulfide-bonded species of α1(I) chains (($\alpha 1^{Cys})_2$). The film was overexposed to emphasize the presence of the disulfide-bonded species.

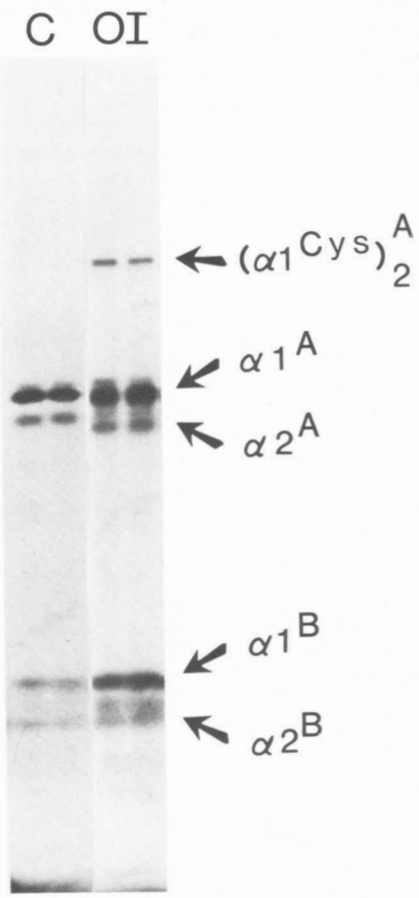

FIG. 2. **SDS-polyacrylamide gel electrophoresis of collagenase cleaved type I collagen from control (*C*) and patient's cells (*OI*).** Cell layer proteins labeled as described in the legend to Fig. 1 were cleaved with vertebrate collagenase and then digested with trypsin and chymotrypsin. Samples were separated without reduction in a gradient gel of 6–14% polyacrylamide. $(\alpha 1^{Cys})^A_2$ is a species of disulfide-bonded $\alpha 1^A$ fragments. The film was overexposed to emphasize the presence of the disulfide-bonded species.

The gel lane was incubated with cyanogen bromide and then the samples were electrophoresed in the second dimension with or without prior reduction (Fig. 3). After reduction, the presumptive dimers of α1(I) chains were found to generate the expected cyanogen bromide fragments. The results demonstrated, therefore, that the disulfide-linked protein did in fact consist of α1(I) chains. However, a distinct band of α1-CB7 was seen only in the reduced sample. Accordingly, the new cysteine residue that generated dimers was located in α1-CB7, a fragment containing amino acid residues 551–822 of the α1(I) chain. Since the cysteine was in the collagenase A-fragment (Fig. 2), the cysteine was located between residue 551 and 775, the cleavage site of vertebrate collagenase.

Cloning and Sequencing of the Region of the cDNA Containing the Mutation—To define the mutation further, a cDNA library was prepared with mRNA from the proband's fibroblasts. To obtain cDNAs for the appropriate region of the pro-α1(I) chain, a specific oligonucleotide priming the 3′coding sequences of the chain was used (see "Materials and Methods"). The clones were screened with a cDNA for the human pro-α1(I) chain and further screened for the presence of a *Bam*HI site found near the vertebrate collagenase cleavage site (Fig. 4). Three positive clones were subcloned into the filamentous phage M13mp18 (Fig. 4). Dideoxynucleotide sequencing with reverse transcriptase revealed that a thymine had replaced a guanine in the coding sequence at amino acid position 748 of the α1(I) chain (Fig. 5). The single-base substitution converted a glycine codon to a cysteine codon. Two independently isolated clones gave the same sequence, indicating that the base change was not an artifact of cDNA synthesis or cloning. The third clone had an identical sequence to that of the published sequence for the same region of the normal cDNA (38), an observation confirming the heterozygous nature of the defect.

Thermal Stability of the Proband's Type I Collagen—To establish that the mutation changed the functional properties of the type I procollagen synthesized by the proband's fibroblasts, the helical stability of the procollagen was examined by controlled temperature digestion with a mixture of trypsin and chymotrypsin. Also, to determine whether the proband's parents had the same mutation, procollagen from the parents was examined in the same manner. As indicated in Figs. 6 and 7, the type I procollagen from the proband's fibroblasts had a decreased thermal stability. The type I procollagen that did not generate disulfide-linked α1(I) chains had a biphasic melting curve (Fig. 7*B*). About 80% of the α chains had an apparent T_m of 39 °C, whereas the remainder had a T_m of about 42 °C, the same value as procollagen from control cells. The procollagen that generated disulfide-linked α1(I) chains had a T_m of about 38 °C (Fig. 7*C*).

In parallel experiments, type I procollagen from the parent's fibroblasts were shown to have normal stability and did not generate disulfide-linked α1(I) chains (Fig. 6).

Secretion of Procollagen—The secretion of type I procolla-

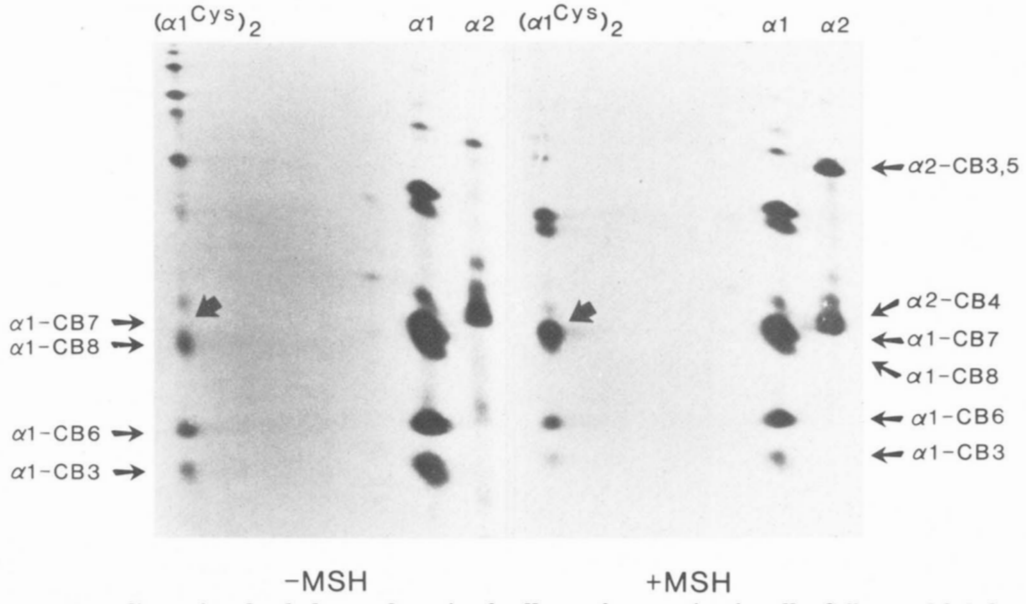

FIG. 3. **Two-dimensional gel electrophoresis of collagen from patient's cells.** Cells were labeled and α chains were separated by gel electrophoresis as described in the legend to Fig. 1. Individual lanes from the gel were cut out, incubated with CNBr, and electrophoresed in a 10% polyacrylamide gel in a second dimension in the presence (+*MSH*) or absence (−*MSH*) of 2-mercaptoethanol. *Heavy arrows* show the position of α1-CB7, which appears only after reduction.

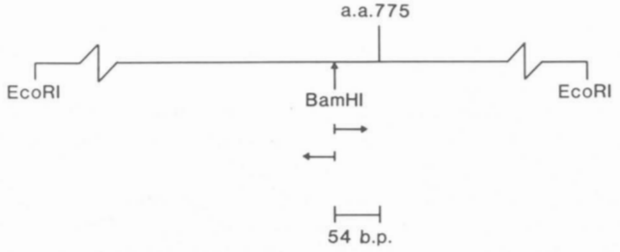

FIG. 4. **Map of cDNA clones.** Poly(A)-enriched mRNA was isolated from the patient's cells and used to construct a cDNA library. Shown are the synthetic *Eco*RI sites used for cloning into λ gt10. Also shown is the location of the collagenase cleavage site at amino acid 775. *Arrows* show the region sequenced.

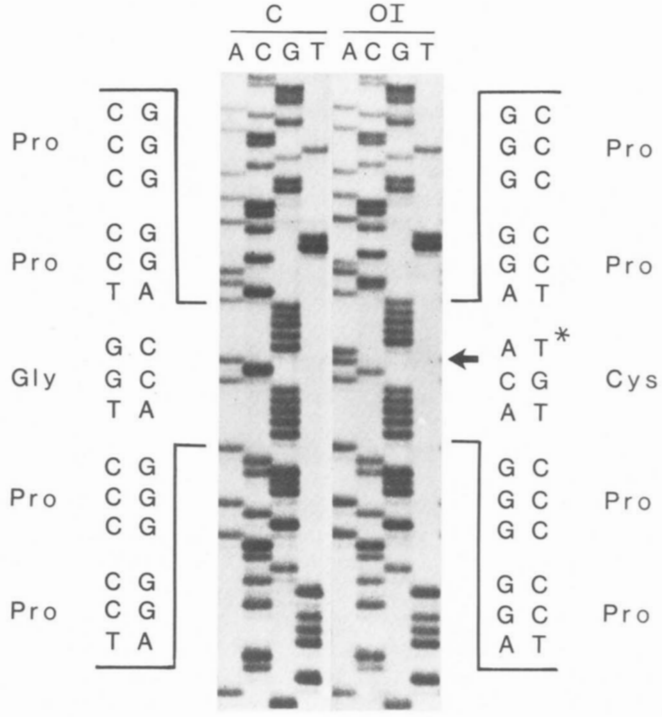

FIG. 5. **DNA sequence of normal (*C*) and mutant (*OI*) cDNA clones.** cDNA clones were sequenced from the *Bam*HI site (Fig. 4) using reverse transcriptase. The *arrow* marks the site of the lesion. *Brackets* indicate borders of sequence shown. Also shown is the complementary coding sequence and the encoded amino acids.

gen by the proband's fibroblasts was examined with two experimental protocols.

In initial experiments, cells were labeled for 4 h with [^{3}H] proline and the label was chased using a protocol employed previously (19, 40). The results suggested that there was a decrease in the rate of secretion of type I procollagen, particularly at short chase times. The differences from control, however, were small (not shown).

Subsequently, experiments were carried out in which normal and proband cultures were labeled continuously for up to 8 h (21). The proteinase-resistant α chains in the medium and cell layers were then assayed as a measure of the rate of secretion of procollagen. As indicated in Fig. 8, the proband's fibroblasts secreted type I procollagen more slowly than control fibroblasts. Again, the difference from control was most marked at the early time points. The disulfide-linked dimers were secreted even more slowly than the bulk of the type I procollagen. Since the cell layer of control fibroblasts labeled for several hours includes procollagen that is secreted and processed to collagen (see Ref. 41), the difference in rate of secretion may be even larger than suggested by the data in Fig. 8.

Processing of Type I Procollagen—To examine further the functional consequences of the mutation, the proband's fibroblasts were grown to postconfluency, and then pulse-labeled and chased with a protocol recently developed to study the processing of type I procollagen (41).

As indicated in Fig. 9*A*, control fibroblasts processed most of the pro-α chains of type I procollagen in the cell layer to collagen α chains during the 8-h chase period. In contrast, there was essentially no processing of type I procollagen in the cell layer of the proband's fibroblasts, apparently because these chains were not secreted (see Fig. 8).

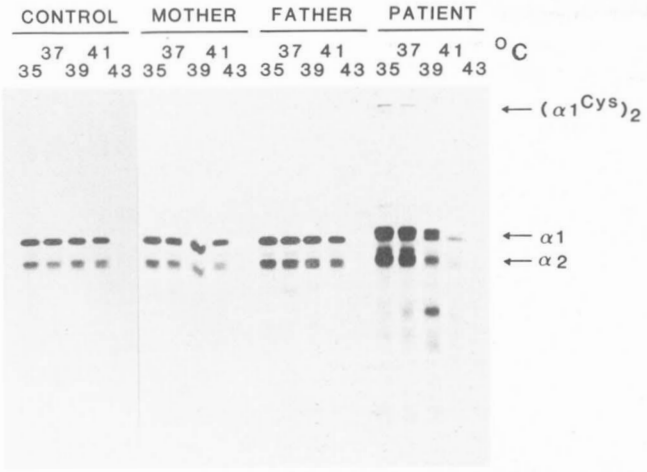

FIG. 6. **Thermal denaturation of type I procollagen.** Samples were incubated for 10 min at the indicated temperatures and then digested briefly with trypsin and chymotrypsin. The *lanes* containing the patient's samples were overexposed to allow visualization of the α1 dimers $(\alpha 1^{Cys})_2$.

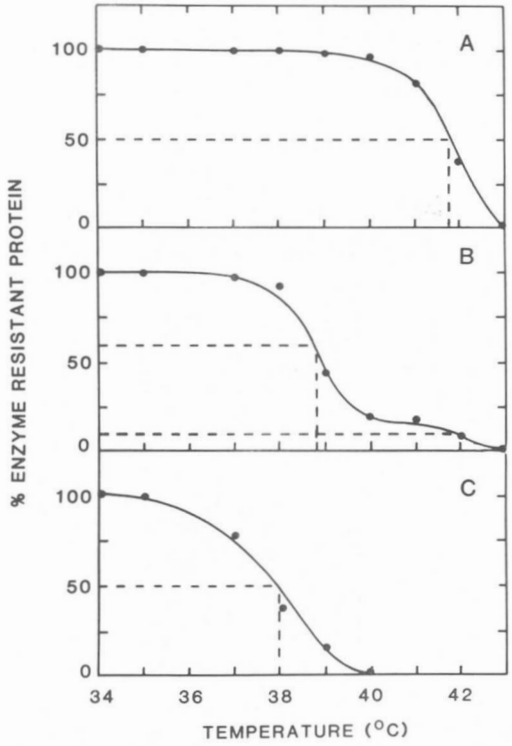

FIG. 7. **Melting curves of type I procollagen from control and OI fibroblasts.** Values for the relative resistance of type I procollagen to proteinase digestion at different temperatures were obtained by densitometry of fluorograms such as those shown in Fig. 6. *Panel A,* thermal stability of type I procollagen from control fibroblasts (T_m about 42 °C). *Panel B,* thermal stability of patient type I procollagen that did not generate disulfide-linked α1(I) chains. Two populations of molecules are present. One has a T_m of approximately 39 °C and the second has a T_m close to that of the control (42 °C). *Panel C,* thermal stability of a population of molecules generating α1(I) dimers (T_m about 38 °C).

Examination of the medium in the same experiments indicated that there was a defect in processing of the procollagen to pC collagen by *N*-proteinase (Fig. 9*B*). In control fibroblasts, approximately 50% of the type I procollagen was cleaved to pCα or α chains after 8 h into the chase period. Values of 40–70% were previously obtained with other normal human fibroblasts (41). In contrast, less than 20% of the type

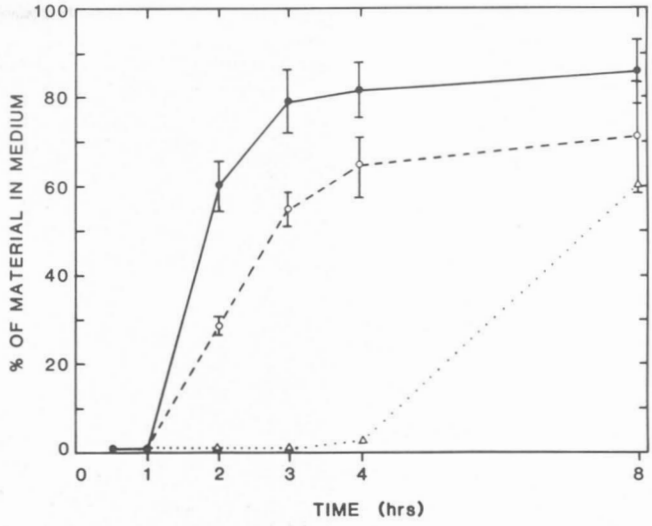

FIG. 8. **Secretion of type I procollagen from control and OI fibroblasts.** Cells from control and patient fibroblasts were labeled for the time indicated. At each time point, the medium and cell layer were collected, digested with trypsin and chymotrypsin, and run on 6% polyacrylamide gels. The amount of α chains in the medium was compared to the total amount of α chains by scanning densitometry of fluorograms. The amount of α chain material secreted by the OI fibroblasts (○) was slightly less than that of the control cells (●). No disulfide-bonded material (△) was found in the medium until 4 h of labeling time.

I procollagen in the medium of the proband's fibroblasts was processed (Fig. 9*B*).

Relative Amount of Disulfide-linked Dimer—In further experiments, we determined the percent of the total pro-α1(I) chains that was recovered as disulfide-linked dimers of α1(I) chains after protease digestion. The proband's fibroblasts were labeled with [^{3}H]proline for 0.5, 1, 2, 3, 4, or 8 h, and the proteins were processed and analyzed as described in the legend to Fig. 1. Fluorograms exposed for varying times were then analyzed by densitometry (Fig. 10). Comparison of peak areas indicated that the ratio of disulfide-linked dimer to free α1(I) chains varied from 4.6 to 9.9% for the cell layer (Fig. 10*A*). Therefore, the results indicated that not more than 10% of the pro-α1(I) chains were disulfide-linked under conditions in which 80% of the protein had a decreased thermal stability (see Fig. 7). In the medium, the ratio of disulfide-linked α1(I) chains to free α1(I) chains was consistently less than 3% (Fig. 10*B*).

DISCUSSION

Mutations that change the structure of type I procollagen are of interest from two points of view. One is that they explain the molecular basis of heritable disorders of connective tissue such as OI and EDS. The other is that the mutations provide a means of establishing the structure-function relationships of the procollagen molecule.

The data presented here provide direct evidence for the previous suggestion (21, 22) that a single point mutation that converts a glycine residue in the α1(I) chain of type I collagen to a cysteine residue can produce a lethal variant of OI. In the proband, a point mutation converted the glycine residue at amino acid position 748 of the α1 chain to a cysteine. The substitution of cysteine for glycine was accompanied by a marked decrease in the thermal stability of the procollagen and decreases in both the rate of secretion and the processing of the NH_2-terminal propeptide. Both parents were phenotypically normal and their fibroblasts did not synthesize a type I procollagen containing the additional cysteine residue. Also,

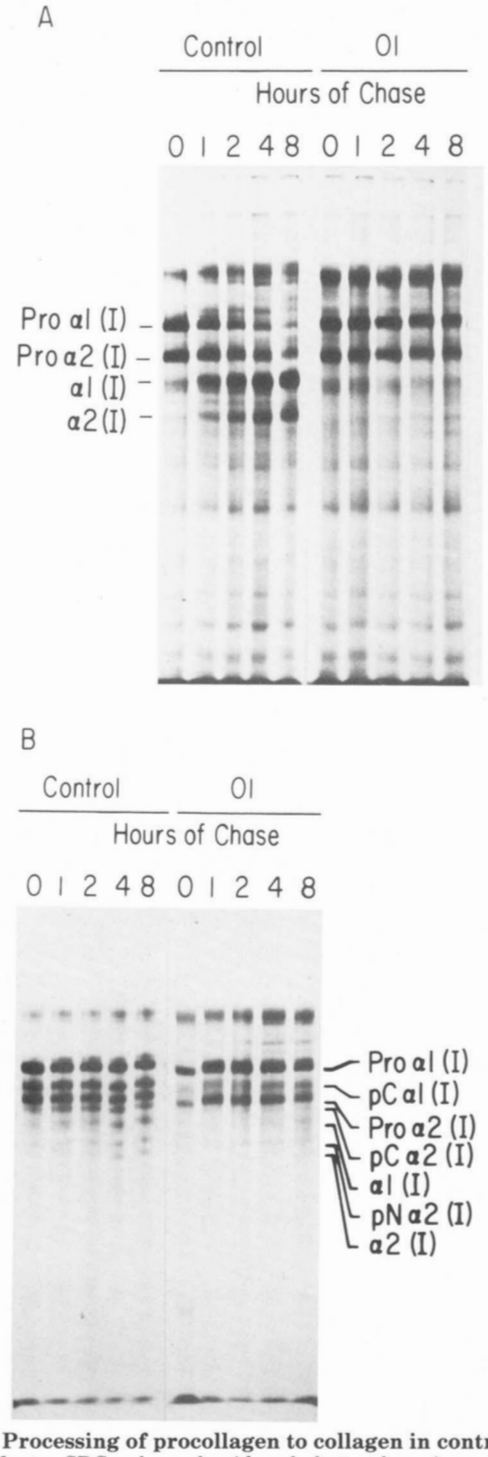

FIG. 9. **Processing of procollagen to collagen in control and OI fibroblasts.** SDS-polyacrylamide gel electrophoresis analysis of ^{3}H-labeled proteins in the cell layer (*Panel A*) and culture medium (*Panel B*) of skin fibroblasts grown 4 days postconfluence, fed for 2 days with medium containing ascorbate, labeled with medium containing [^{3}H]proline, and chased for 0, 1, 2, 4, and 8 h with medium without label (see "Materials and Methods"). *Panel A* shows that in the cell layer of control fibroblasts over 90% of the pro-α chains were processed to α chains, whereas in the cell layer of the OI fibroblasts less than 10% of the pro-α chains were processed. *Panel B* shows that in the culture medium of control fibroblasts about 50% of the pro-α chains were processed to pCα, pNα, or α chains, while in medium of OI fibroblasts about 20% of the pro-α chains were processed.

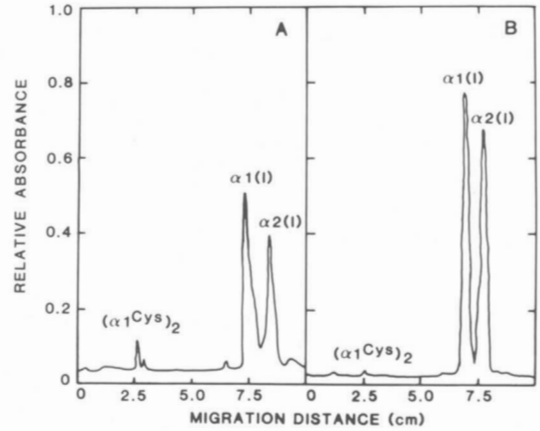

FIG. 10. **Relative amounts of disulfide-linked pro-α1 chains.** OI fibroblasts were incubated with [^{3}H]proline for 4 h. The cell layer and medium protein were digested with a mixture of trypsin and chymotrypsin, and separated by electrophoresis on 6% polyacrylamide gels. Fluorograms of the gels were then scanned. *A*, cell layer; *B*, medium.

the procollagen synthesized by the parental fibroblasts had a normal thermal stability and was not post-translationally overmodified. Therefore, the mutation introducing a cysteine residue was a sporadic one. Also, it is very likely that the mutation was the direct cause of lethal phenotype in the proband. A similar conclusion was suggested by the data on the lethal variant studied by Steinmann *et al.* (21) and Cohn *et al.* (22) in which the glycine residue in amino acid position 988 of the α1(I) chain was converted to cysteine. In those studies, however, the family inherited the Marfan syndrome as a dominant phenotype and it was not clear whether the Marfan allele contributed to the lethal OI phenotype (21).

Data are now available on four OI variants in which a new cysteine residue is introduced into the α1(I) chain (Table I). In both the lethal variant studied here and the lethal variant studied by Steinmann *et al.* (21) and Cohn *et al.* (22), the substitution of a cysteine residue for a glycine residue decreased the thermal stability of the procollagen by 3–4 °C. Also, there was a similar decrease in the rate of secretion of procollagen. The decreased processing of the N-propeptide seen here was not examined by Steinmann *et al.* (21) or Cohn *et al.* (22). We have recently shown that a fraction of purified type I procollagen from the proband studied here is resistant to cleavage by partially purified type I procollagen *N*-proteinase.[3] Therefore, the decrease in processing of the N-propeptide is explained by an alteration in the structure of the protein and not by a secondary effect on enzyme activity or other factors that might alter processing in cultured fibroblasts. Type I procollagen *N*-proteinase has a highly specific requirement for procollagen in a native conformation and will not cleave isolated pro-α chains or partially unfolded procollagen (42, 43). The decrease in processing of type I procollagen indicates, therefore, that the presence of a cysteine residue in amino acid position 748, and 766 residues from the cleavage site of the *N*-proteinase, has a long-range effect on the conformation of the site.

As suggested previously (19, 20), the two OI variants in which the presence of a cysteine residue in the α1(I) chain did not produce a lethal phenotype (Table I) may well be mutations that change amino acids other than glycine to a cysteine. Since the glycine residue found as every third amino acid in collagen α chains must fit into the confined space in

[3] B. Vogel, K. Kadler, Y. Hojima, and D. Prockop, manuscript in preparation.

TABLE I

Comparison of OI variants containing a cysteine residue in the α1(I) chain

Phenotype	Mutation	Relative amount of dimer	Decrease in T_m[a]	Secretion of procollagen[b]	*N*-Propeptide processing
Lethal OI[c]	$Gly^{988} \rightarrow Cys^{988}$	Variable	3–4 °C	Decreased	Not assayed
Lethal OI[d]	$Gly^{748} \rightarrow Cys^{748}$	6–10%	3–4 °C	Decreased	Decreased
Moderate OI[e]	$? \rightarrow Cys^{123-402}$	25%	1.5–2.5 °C	Decreased	Normal
Moderate OI[f]	$? \rightarrow Cys^{817-1014}$	25%	Normal	Normal	Not assayed

[a] In the two lethal variants, procollagen protomers containing the disulfide-linked pro-$\alpha 1^{Cys}$ chains had slightly lower T_m than protomers containing one or two pro-$\alpha 1^{Cys}$ chains that were not disulfide-linked. The reverse was true for the moderate variant studied by de Vries and de Wet (19).

[b] Defects in secretion with the two lethal variants appeared similar in that control fibroblasts secreted 80–86% in 4–6 h in continuous labeling experiments, whereas the values were 41–65% with the OI fibroblasts (see Fig. 8 and Refs. 20 and 21). The decreased secretion in one of the moderate OI variants was detected in a pulse-chase experiment and, therefore, the results are not directly comparable.

[c] Cohn *et al.* (22); Steinmann *et al.* (21).

[d] Variant studied here.

[e] de Vries and de Wet (19).

[f] Steinmann *et al.* (20).

the center of the triple helix, mutations that replace glycine with cysteine are likely to have more marked effects on the thermal stability of the protein and on the phenotype than mutations that change other amino acids in type I collagen to cysteine (44). This hypothesis, however, may well be too simple. For example, it is not obvious why one proband with a moderately severe form of OI had a cysteine substitution that decreased the thermal stability of the procollagen (19), whereas another proband with a mild form of OI had a cysteine substitution that did not alter the thermal stability of the protein (20).

In the lethal variant studied here, 80% of the type I procollagen in the cell layer after labeling for 4 h had a decreased thermal stability. As noted previously (6), this observation suggests that approximately equal amounts of abnormal and normal pro-α1(I) chains are synthesized and that the chains are randomly incorporated into protomers so that about 50% contain one abnormal pro-α1(I) chain and about 25% contain two abnormal pro-α1(I) chains. Similar observations and conclusions were made concerning the two non-lethal variants with new cysteine residues (19, 20). In the present variant, however, only 10% of the pro-α1(I) chains in the cell layer after labeling for 4 h were disulfide linked. In contrast, the value was about 25%, or near the theoretical maximal, in the two non-lethal variants with new cysteine residues (19, 20). The lower value here suggests that the cysteine in position 748 is more hindered in formation of interchain disulfide bonds than cysteine residues at other sites in the α1(I) chain.

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