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**Research Article**

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# Osteogenesis Imperfecta Type IV

## Biochemical Confirmation of Genetic Linkage to the pro $\alpha$ 2(I) Gene of Type I Collagen

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### Abstract

Fibroblasts from two affected members of a large pedigree in which osteogenesis imperfecta (OI) type IV is genetically linked to the pro $\alpha$ 2(I) gene of type I collagen synthesize two populations of pro $\alpha$ 2(I) chains. One population is normal; the second population appears to have a deletion of about 10 amino acid residues from the middle of the triple helical domain. The mutation in pro $\alpha$ 2(I) causes increased posttranslational modification in the amino-terminal half of some pro $\alpha$ 1(I) chains, lowers the melting temperature of type I collagen molecules that incorporate a mutant pro $\alpha$ 2(I) chain, and prevents or delays the secretion of those molecules from fibroblasts in cell culture. On the basis of this study and linkage studies in additional families, it appears that the OI type IV phenotype is often the result of heterozygosity for mutations in pro $\alpha$ 2(I) that alter the triple helical structure of type I collagen.

### Introduction

Most forms of osteogenesis imperfecta (OI)<sup>1</sup> are due to structural abnormalities in or decreased production of type I collagen (1–3). Studies of collagens synthesized by cultured fibroblasts from different patients with OI type I (4–6), OI type II (7–17), and OI type III (18–22) have demonstrated evidence of mutations in the pro $\alpha$ 1(I) and pro $\alpha$ 2(I) genes of type I collagen. There is less information regarding the biochemical basis of OI type IV, which differs clinically from the other relatively mild autosomal dominant OI phenotype, OI type I. Individuals with OI type IV have normal or gray rather than blue sclerae, may have mild to moderate short stature with long bone deformity, and have a far greater incidence of dentinogenesis imperfecta (23, 24). We have recently provided evidence of abnormal triple helical structure of type I collagen in one patient with the OI type IV phenotype (25), and Tsipouras et al. have demonstrated linkage of the OI type IV phenotype to the pro $\alpha$ 2(I) gene of type I collagen on chromosome 7 in four pedigrees (26–28).

To circumvent some of the difficulties inherent in the analysis of subtle abnormalities in a large heteropolymeric molecule such

as type I collagen, we chose to study cell strains from individuals in a large pedigree in which there is evidence of linkage of the OI type IV phenotype to the pro $\alpha$ 2(I) gene (26). This approach enabled us to focus attention on only one class of constituent chains of type I collagen. In this report we describe studies of type I collagen synthesized by cells cultured from one unaffected and two affected members of this pedigree (26). The affected members are heterozygous for a mutation in the pro $\alpha$ 2(I) gene that produces a shortened pro $\alpha$ 2(I) chain, thus confirming linkage in this family. The deletion from the middle of the triple helical domain of  $\alpha$ 2(I) causes excessive posttranslational modification of the amino-terminal half of the  $\alpha$ -chains in molecules that contain the shortened  $\alpha$ 2(I) chains. Molecules that contain the mutant  $\alpha$ 2(I) chains are selectively retained within fibroblasts. These studies provide direct evidence that mutations in  $\alpha$ 2(I) are responsible for the clinical and biochemical features of OI type IV in this family and, in the context of additional linkage data (27, 28) and biochemical studies (reference 25, Wenstrup, R. J., and P. H. Byers, unpublished observations), suggest that many individuals with OI type IV have mutations in  $\alpha$ 2(I).

### Methods

Fig. 1 shows the pedigree. Cells from one unaffected member (II-3) and two affected family members (II-5 and III-6) were studied. The details of physical findings for II-5 and III-6 are presented here and Table 1 summarizes the findings in all family members.

*II-5.* A 41-yr-old mother of the proband III-6 who sustained eight fractures during childhood and adolescence. Her first fracture was at the age of 2 yr. The diagnosis of OI was established when her son was born. On physical examination her measurements were: height 153 cm (3%), weight 53.2 kg (50%), and head circumference 57.4 cm (97%). No skeletal deformity was observed. Scleral hue was white, teeth were discolored, there was radiologic evidence of dentinogenesis imperfecta, and the audiogram revealed a normal range of hearing. Patient gave a history of easy bruising.

*III-6.* An 8-yr-old male born with rib fractures, who subsequently sustained approximately 25 fractures. At birth he was noted to have bowed legs. On physical examination his measurements were: height 113.3 cm (<3%), weight 22.4 kg (25%), and head circumference 53 cm (>97%). An angulation deformity of the right tibia was observed but there was no scoliosis or pectus deformity. Scleral hue was white. Teeth in mixed dentition were discolored. There was radiologic evidence of dentinogenesis imperfecta and the audiogram revealed a normal range of hearing.

Dermal fibroblasts were obtained from outgrowths of skin taken from the inner aspect of the forearm of two affected and one unaffected family members (II-3, II-5, and III-6 in the pedigree). Biochemical studies were performed on cells between the fourth and twelfth passages. The cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) (Gibco, Grand Island, NY), 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 2.5 mM glutamine in a humidified atmosphere of 9% CO<sub>2</sub>/air at 37°C. Control cell strains were obtained from newborn individuals who had no evidence of connective tissue disease. All samples were obtained with appropriate consent.

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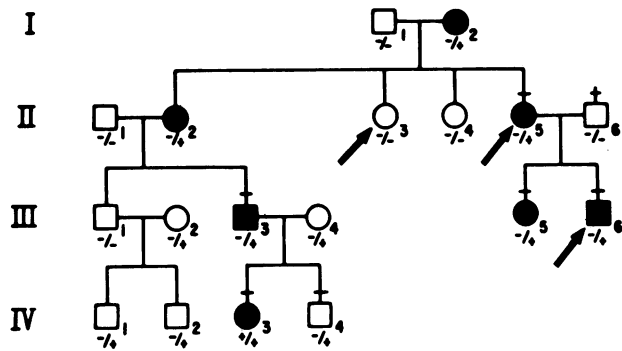
1. Abbreviations used in this paper: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; OI, osteogenesis imperfecta; PAGE, polyacrylamide gel electrophoresis.

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**Figure 1.** Family pedigree, reprinted with permission (26). Symbols: individuals heterozygous (-/+) and homozygous for the absence (-/-) and presence (+/+) of a polymorphic EcoRI site in  $\alpha 2(I)$ ; horizontal bars, individuals examined by one of us (Dr. P. Tsipouras); solid circles, solid squares, affected by OI; arrows, studied biochemically in this paper.

To label proteins for analysis of procollagens or collagens,  $2.5 \times 10^5$  cells were plated in 35-mm culture dishes (Corning Science Products, Corning, NY) and allowed to attach and spread overnight. Unless otherwise indicated, the cells were preincubated for 4 h in 0.7 ml DMEM lacking FCS and supplemented with 50  $\mu\text{g}/\text{ml}$  ascorbic acid. The proteins were radiolabeled by addition of  $[2,3,4,5\text{-}^3\text{H}]\text{-proline}$  (108 Ci/mmol; Amersham Corp., Arlington Heights, IL) at concentrations of 30 to 120  $\mu\text{Ci}/\text{ml}$ , depending on the studies planned. After 16 h incubation, medium and cell layer proteins were harvested separately and proteolytic enzymes were inhibited as described (7). Samples were concentrated by precipitation with 30% ethanol followed by air drying. Medium and cell layer procollagens were analyzed under reducing conditions on 5% sodium dodecyl sulfate (SDS) polyacrylamide gels as described by Laemmli (29) except that 2 M urea was included in the system to enhance the separation of  $\text{pro}\alpha 1(I)$  from  $\text{pro}\alpha 1(III)$  chains. Dried procollagens were dissolved in 60  $\mu\text{l}$  of sample buffer with 7.7 mg/ml dithiothreitol and boiled for 3 min to reduce disulfide bonds and denature the procollagen molecules. After electrophoresis, radioactive proteins were detected by radio-autofluorography (30) using EN<sup>3</sup>HANCE (New England Nuclear, Boston, MA) as the fluor.

To examine ratios of newly synthesized  $\text{pro}\alpha 1(I)$  to  $\text{pro}\alpha 2(I)$  chains,  $1.5 \times 10^5$  cells were plated in 35-mm culture dishes, allowed to spread overnight, and then preincubated for 4 h as described above. The cells were trypsinized off the dishes into microcentrifuge tubes, pelleted at 10,000 rpm for 5 s, and the supernatant was removed. 30  $\mu\text{l}$  of the preincubation medium was added, the cells were gently resuspended, and  $[2,3,4,5\text{-}^3\text{H}]\text{-proline}$  was added to a concentration of 2 mCi/ml for 18 min. Then the cells were repelleted, the medium discarded, and the cells were washed once with phosphate-buffered saline. The cells were suspended in sample buffer containing 2% SDS and 2 M urea and boiled

for 5 min. Intracellular  $\text{pro}\alpha$  chains were separated by electrophoresis in a 5% SDS polyacrylamide gel as described above. Quantitation of radioactivity in  $\text{pro}\alpha 1(I)$  and  $\text{pro}\alpha 2(I)$  chains was performed by densitometry of autoradiofluorograms exposed in the linear range (31).

The ratio of type I to type III secreted collagen into culture medium during a 16-h incubation was measured by gel densitometry of fluorograms exposed in the linear range (31). The density of all the  $\text{pro}\alpha$ ,  $\text{pC}\alpha$ ,  $\text{pN}\alpha$ , and  $\alpha$  chains was determined and summed for each collagen. Incorporation of  $[^3\text{H}]\text{-proline}$  into collagenous protein was measured by bacterial collagenase digestion as previously described (32).

To examine unmodified  $\text{pro}\alpha$  chains,  $2.5 \times 10^5$  cells were plated as above and then were preincubated for 4 h in 0.7 ml DMEM that lacked FCS but contained 50  $\mu\text{g}/\text{ml}$  of ascorbic acid and 0.5 mM  $\alpha, \alpha'$ -dipyridyl. The cells were then incubated with 100  $\mu\text{Ci}/\text{ml}$  of  $[^3\text{H}]\text{-proline}$  for 16 h under identical conditions. Cell layer proteins were harvested and analyzed by electrophoresis under reducing conditions as described above.

Collagens were prepared by dissolving ethanol-precipitated procollagens in 0.5 N acetic acid and digesting with 50  $\mu\text{g}/\text{ml}$  pepsin (Boehringer Mannheim Diagnostics, Houston, TX) at 4°C for 16 h. The reaction was terminated by adding pepstatin to 5  $\mu\text{g}/\text{ml}$  and molecules were concentrated by lyophilization and analyzed by electrophoresis.

Fibroblast collagenase fragments of  $\alpha 1(I)$  and  $\alpha 2(I)$  chains were prepared from  $[^3\text{H}]\text{-proline}$ -labeled procollagens after digestion with pepsin (33). The fibroblast collagenase was a generous gift from Eugene Bauer, M.D. (Washington University, St. Louis, MO).

CNBr cleavage of  $\alpha$ -chains and of fibroblast collagenase products of  $\alpha$ -chains of type I collagen in gels was performed as previously described (34) except that digestion was limited to 2 h and the samples were subsequently washed three times with 20 vol of water for 10 min each (13). The peptides were separated on second-dimension gels as described (34).

In some experiments, collagenous proteins were cleaved sequentially by pepsin, fibroblast collagenase, and CNBr. The collagenase fragments were cleaved with 20  $\mu\text{g}/\text{ml}$  CNBr in 70% formic acid at 30°C for 5 h, lyophilized, washed in distilled  $\text{H}_2\text{O}$ , and lyophilized again. The peptides were separated by isoelectric focusing in a vertical slab gel using the gel composition and buffers previously described (35). After first-dimension electrophoresis, gel strips were cut out and washed in 30 vol of water for 20 min three times, then twice in 15 vol of 30% glycerol, 0.1 M Tris-HCl (pH 6.8) for 10 min, and loaded onto a 12.5% SDS polyacrylamide gel with a 5% stacking gel for separation of peptides in the second dimension.

Thermal denaturation temperatures of normal and abnormal collagens were determined as previously described (36).

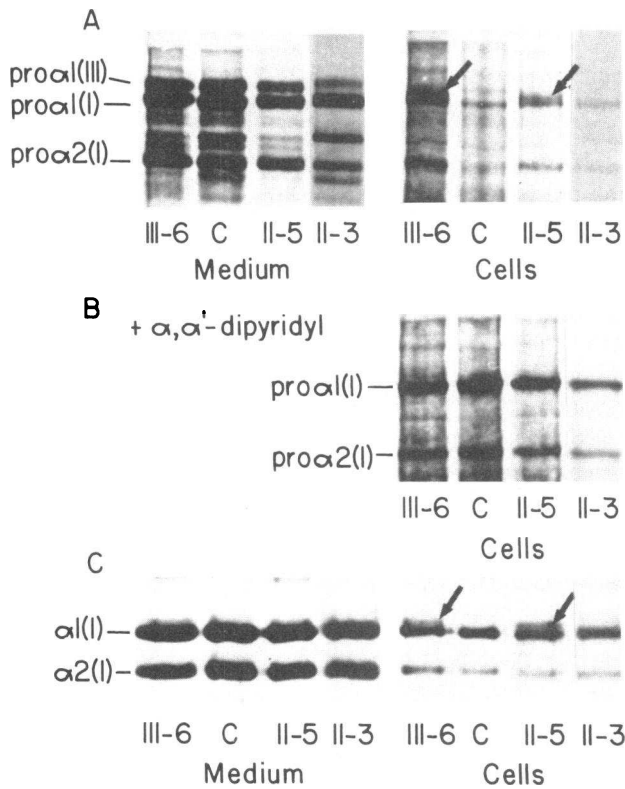
## Results

Dermal fibroblasts from two affected members of this family synthesized two populations of  $\text{pro}\alpha 1(I)$  chains of type I procollagen. One population co-migrated with the corresponding  $\text{pro}\alpha 1(I)$  chains synthesized by cells from the control and from

**Table I.** Clinical Features of Affected Examined Members of Family with OI Type IV

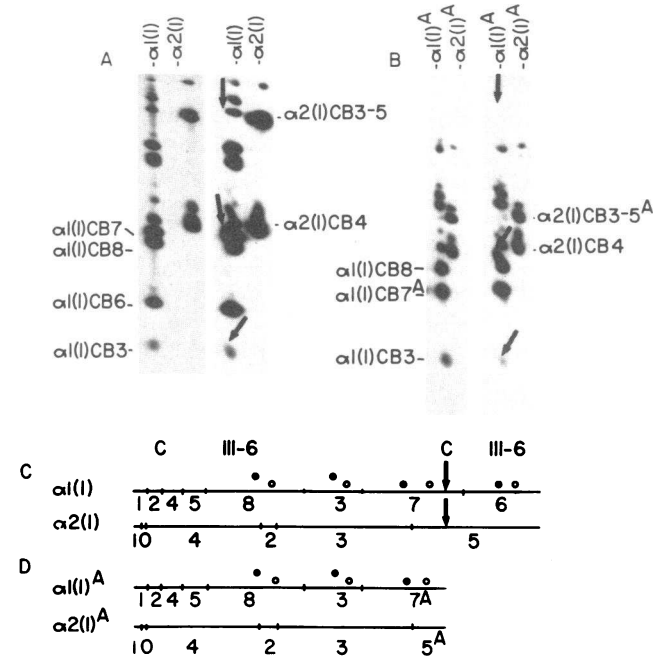
Pedigree	Age	Fractures	Height	Skeletal deformity	Scleral hue	Dentinogenesis imperfecta	Hearing
	yr		cm				
I-2	75	None	133 (<3%)	None	White	Edentulous	Mixed loss
II-2	57	1	149.5 (3%)	None	White	Present	Sensorineural
II-5	41	8	153 (10%)	None	White	Present	Normal
III-3	29	20	156.5 (<3%)	None	White	Present	Not tested
III-5	18	3	150.2 (3%)	Angulation right elbow	White	Present	Normal
III-6	8	25	113.3 (<3%)	Angulation right tibia	White	Present	Normal
IV-3	3	7	81.5 (<3%)	None	White	Present	Normal

an unaffected family member. A second population, seen only in the cell layer, migrated more slowly and appeared as a broad band or doublet during electrophoresis (Fig. 2 A). The slower migrating pro $\alpha$ 1(I) chains were from within the cells, for when cells were removed from the matrix by trypsinization before isolating the pro $\alpha$  chains, the abnormal chains were still present (data not shown). When pro $\alpha$  chains were labeled in the presence of  $\alpha, \alpha'$ -dipyridyl, which inhibits posttranslational modification of prolyl and lysyl residues, the OI cells produced only a single population of pro $\alpha$ 1(I) chains, which co-migrated with pro $\alpha$ 1(I) chains synthesized by control cells and those from the unaffected family member (Fig. 2 B). This indicated that the apparent increase in molecular weight of the slower migrating pro $\alpha$ 1(I) chains found inside OI cells was due to increased posttranslational modification, not peptidyl insertion. To determine whether the increased posttranslational modifications involved the triple helical domain,  $\alpha$ -chains produced by pepsin digestion of procollagens were prepared and separated by electrophoresis (Fig. 2 C). There were two populations of  $\alpha$ 1(I) from the cell layer



**Figure 2.** Autoradiofluorograms of radiolabeled procollagens after SDS PAGE (A) Pro $\alpha$  chains from medium and cell layer. There was a slower migrating population of pro $\alpha$ 1(I) chains (arrows) from the cell layer of OI cell strains (II-5 and III-6) not seen in control (C) or the unaffected family member (II-3). Only normally migrating pro $\alpha$  chains appeared in medium of OI and control cell strains. (B) Unmodified, intracellular pro $\alpha$  chains from OI and control cell strains produced by labeling in the presence of  $\alpha, \alpha'$ -dipyridyl. OI cell strains (II-5 and III-6) produced only single populations of pro $\alpha$ 1(I) chains that co-migrated with those of control. Fibroblasts treated with  $\alpha, \alpha'$ -dipyridyl did not secrete significant amounts of procollagen into the fibroblast medium. (C) Collagenous proteins after partial proteolytic digestion with pepsin to yield  $\alpha$ -chains. There was a slow migrating population (arrows) of  $\alpha$ 1(I) in the cell layer of OI cell strains (II-5 and III-6) not seen in controls. Slow migrating  $\alpha$ 1(I) chains were not seen in medium from OI fibroblasts.

samples from the patients' cells but single populations from the cell layers of control and the unaffected family member cells. Medium from OI fibroblasts contained single populations of  $\alpha$ 1(I) chains that co-migrated with the  $\alpha$ 1(I) chains made by control cells and cells from the unaffected family member. Thus, the increased posttranslational modification of intracellular pro $\alpha$ 1(I) chains involved the triple helical domain. To determine which region of the slower migrating  $\alpha$ 1(I) chains from OI cells was overmodified, CNBr peptides of normal and slow migrating chains were examined (Fig. 3).  $\alpha$ -Chains were separated in the



**Figure 3.** (A) Autoradiofluorogram of CNBr cleavage products of intracellular  $\alpha$ -chains from control (C) and OI (III-6) fibroblasts. Radiolabeled  $\alpha$ -chains were separated on a 5% polyacrylamide gel, then cleaved in the gel with CNBr as described in Methods. The peptides were then separated in a 12.5% gel.  $\alpha$ 1(I) chains from control cells and the normally migrating  $\alpha$ 1(I) chains from the OI cells yielded the usual four large peptides:  $\alpha$ 1(I)CB8,  $\alpha$ 1(I)CB3,  $\alpha$ 1(I)CB7, and  $\alpha$ 1(I)CB6 (see C), as well as partial cleavage products. Slow migrating  $\alpha$ -chains from OI cells (arrow at top of autoradiofluorogram) yielded  $\alpha$ 1(I)CB6, a peptide that migrated more slowly than  $\alpha$ 1(I)CB3 (arrow), a single spot in the region of  $\alpha$ 1(I)CB7 (arrow), and partial cleavage products. (B) Autoradiofluorogram of CNBr peptides of  $\alpha$ 1(I)<sup>A</sup> and  $\alpha$ 2(I)<sup>A</sup>. Radiolabeled  $\alpha$ 1(I)<sup>A</sup> and  $\alpha$ 2(I)<sup>A</sup> fragments generated by fibroblast collagenase cleavage of  $\alpha$ -chains of type I collagen (see Methods) were separated in an 8% polyacrylamide gel, cleaved in the gel with CNBr, and the resulting peptides were separated in a 12.5% gel.  $\alpha$ 1(I)<sup>A</sup> fragments from control cells and the normally migrating  $\alpha$ 1(I)<sup>A</sup> fragments from the OI cells yielded the expected three large peptides:  $\alpha$ 1(I)CB8,  $\alpha$ 1(I)CB3, and  $\alpha$ 1(I)CB7<sup>A</sup> (see D), as well as partial cleavage products. Slow migrating  $\alpha$ 1(I)<sup>A</sup> fragments from OI cells (arrow at top of autoradiofluorogram) yielded  $\alpha$ 1(I)CB7<sup>A</sup>, a peptide that migrated slower than  $\alpha$ 1(I)CB3 (arrow), a peptide that migrated slower than  $\alpha$ 1(I)CB8 (arrow), and partial cleavage products. (C) Diagram representing the arrangement of CNBr peptides in  $\alpha$ 1(I) and  $\alpha$ 2(I). The arrows in the diagram represent the fibroblast collagenase cleavage sites, and the circles represent the relative mobilities of the normal (open circles) and overmodified (solid circles) peptides. CNBr does not cleave efficiently at the methionine between  $\alpha$ 2(I)CB3 and  $\alpha$ 2(I)CB5. (D) Diagram representing the arrangement of CNBr peptides in  $\alpha$ 1(I)<sup>A</sup> and  $\alpha$ 2(I)<sup>A</sup>. The circles represent the relative mobilities of the normal (open circles) and overmodified (solid circles) peptides.

first dimension by SDS polyacrylamide gel electrophoresis (PAGE) (see Methods), gel strips containing  $\alpha$ -chains were cut out, chains were cleaved in the gel at methionyl residues by CNBr, and the resultant peptides were separated by gel electrophoresis in the second dimension. CNBr peptides of  $\alpha$ -chains from the medium of OI fibroblasts were indistinguishable from control (data not shown). Intracellular  $\alpha 1(I)$  chains from control cells and the normally migrating population of intracellular chains from OI cells yielded the expected four large peptides (Fig. 3 A):  $\alpha 1(I)CB8$ ,  $\alpha 1(I)CB3$ ,  $\alpha 1(I)CB7$ , and  $\alpha 1(I)CB6$ . The slower migrating population of intracellular  $\alpha 1(I)$  chains from OI cells yielded a peptide that migrated slower than  $\alpha 1(I)CB3$ , a normally migrating  $\alpha 1(I)CB6$ , and a peptide that co-migrated with  $\alpha 1(I)CB7$ . Because we thought that the spot corresponding to the  $\alpha 1(I)CB7$  peptide from the slower migrating  $\alpha 1(I)$  chains might also contain slower migrating  $\alpha 1(I)CB8$  peptides, CNBr peptide maps were made from the A fragments generated by fibroblast collagenase, which cleaves  $\alpha 1(I)$  and  $\alpha 2(I)$  asymmetrically (see arrows, Fig. 3 C). Cleavage of  $\alpha 1(I)$  at the collagenase site near the carboxyl end of  $\alpha 1(I)CB7$  followed by CNBr cleavage results in a shortened fragment,  $\alpha 1(I)CB7^A$ , that is easily separated from  $\alpha 1(I)CB8$ . When A fragments of type I collagen retained within OI cells were separated by SDS PAGE and then cleaved with CNBr (Fig. 3 B), the slower migrating  $\alpha 1(I)^A$  fragment produced a peptide that migrated more slowly than  $\alpha 1(I)CB3$ , a normally migrating  $\alpha 1(I)CB7^A$ , and a peptide that migrated more slowly than  $\alpha 1(I)CB8$ . Thus, the slower migrating population of  $\alpha 1(I)$  was overmodified amino-terminal to the  $\alpha 1(I)CB7$  domain.

These findings contained an apparent paradox: although the genetic linkage data (26) indicated that the mutation should be in an  $\alpha 2(I)$  chain, the evident structural abnormality in type I collagen was overmodification of the amino-terminal half of some intracellular  $\alpha 1(I)$  chains produced by the OI cells. Because

Steinmann et al. (12) have demonstrated that a mutation of  $\alpha 1(I)$  could cause overmodification in all three constituent  $\alpha$ -chains in a molecule at residues amino-terminal to the mutation, we hypothesized that a mutation in the middle of an  $\alpha 2(I)$  chain might also cause excessive posttranslational modification amino-terminal to its location in all chains in such a molecule but that because  $\alpha 2(I)$  normally is more modified than  $\alpha 1(I)$ , overmodification in  $\alpha 2(I)$  might be more difficult to detect. Because overmodification of abnormal molecules involved only the amino-terminal half of the triple helical domain, we examined the region in the middle of the triple helical domain of  $\alpha 2(I)$  in more detail. Because the methionyl residue between  $\alpha 2(I)CB3$  and  $\alpha 2(I)CB5$  is inefficiently cleaved by CNBr,  $\alpha$ -chains were cleaved with fibroblast collagenase and then cleaved with CNBr (see diagram, Fig. 3 C, for relative locations of CNBr and fibroblast cleavage sites). Peptides cleaved in a 5% polyacrylamide gel, then gel strips were placed over a 12.5% SDS polyacrylamide gel and peptides were separated by molecular weight in a second dimension (Fig. 4). Although each peptide was heterogeneous with respect to charge as has been reported elsewhere (35, 37), the cell layer samples of the OI cell strains (Fig. 4 B, and lower right, C) contained two major populations of  $\alpha 2(I)CB3-5^A$ . One population was in normal position, and a second population migrated further (vertically) into the second-dimension gel and had a more acidic isoelectric point (shifted leftward in the figure). This second population of  $\alpha 2(I)CB3-5^A$  was not present in collagens secreted by control cells or in the cell layer or secreted collagens of control cells or those from the unaffected family member. These findings are consistent with a small deletion, of about 10 residues, in the  $\alpha 2(I)CB3-5^A$  peptide that involves the net loss of at least one basic residue. Because we did not observe the shortened  $\alpha 2(I)$  fragment in the medium, we determined whether the shortened  $\alpha 2(I)$  chain affected the thermal stability of type I collagen mol-

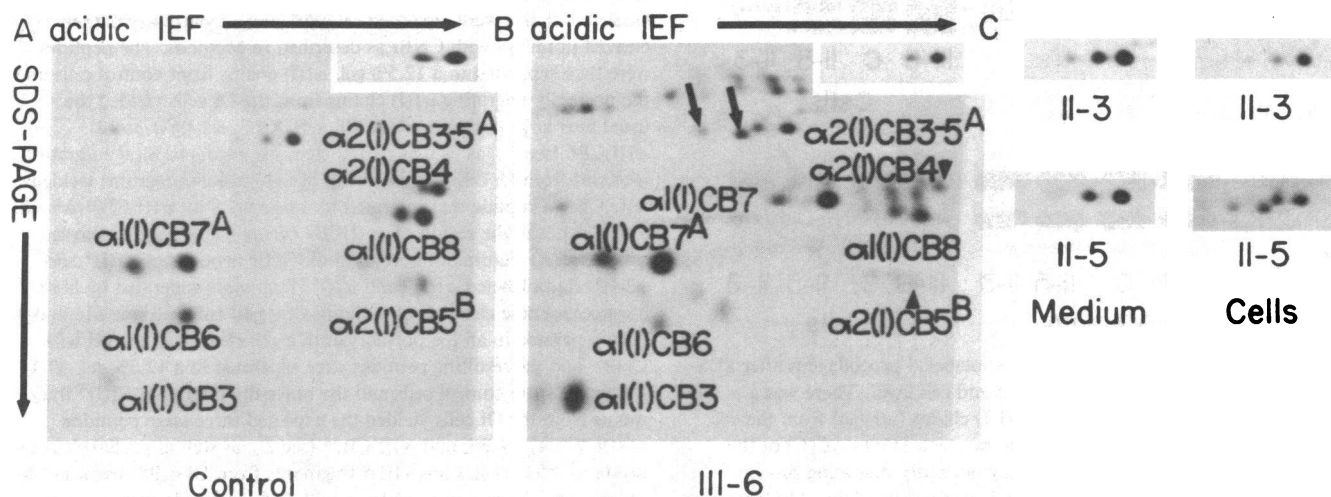
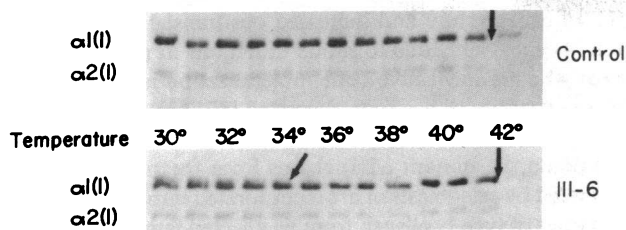


Figure 4. Autoradiofluorograms of two-dimensional maps of peptides generated by cleavage with fibroblast collagenase and CNBr from collagens from control (C) and OI (III-6) cells. Peptides were separated first by isoelectric focusing in a 5% polyacrylamide gel, then separated by molecular weight in a 12.5% SDS polyacrylamide gel in the second dimension. The methionine between  $\alpha 2(I)CB3$  and  $\alpha 2(I)CB5$  is inefficiently cleaved by CNBr. All peptides usually show charge heterogeneity. In B, which shows peptides generated from intracellular collagens from III-6, there was a second, abnormal population of  $\alpha 1(I)CB3-5^A$  which appeared shortened and was more acidic than normal (arrows).

Other peptides from III-6 were normal except for evidence of overmodification of some  $\alpha 1(I)CB8$  and  $\alpha 1(I)CB3$  peptides. The abnormal population of  $\alpha 2(I)CB3-5^A$  was not seen in fibroblast medium from III-6 (inset at top right, B), in the cell layer or medium from control (A and inset in A), or unaffected family member (II-3, C), or in medium from the other affected family member (II-5, C). It was seen in the cell layer of II-5, however (C). The diagram at the bottom of Fig. 3 shows the relative sizes and positions of CNBr peptides and fibroblast collagenase cleavage sites in the triple helical domain of type I collagen.



**Figure 5.** Autoradiofluorogram showing melting temperatures of intracellular type I collagen molecules from control (C) and OI (III-6) fibroblasts. Pepsin-digested samples were gradually warmed from 30° to 43°C at the rate of 1°C/12 min, and samples were removed at 1.0°C intervals, rapidly cooled to 20°C, and then digested with trypsin for 2 min. After trypsin digestion, samples were separated on a 5% SDS polyacrylamide gel. Molecules containing overmodified  $\alpha 1(I)$  chains from OI cells were digested at ~35°C (slanted arrow) as compared with normally migrating type I collagen molecules (vertical arrows) and type I collagen molecules from control cells, which became pro-tease sensitive at 42°C.

ecules that contained them (Fig. 5). Type I collagen from each OI cell strain had a biphasic curve: molecules that contained overmodified  $\alpha 1(I)$  chains (presumably a marker for molecules containing a mutant  $\alpha 2(I)$  chain) melted at 35°C, as compared with molecules that contained normally migrating  $\alpha 1(I)$  and  $\alpha 2(I)$  chains from OI and control cells, which melted at 42°C.

To determine whether the decreased triple helical stability of some type I procollagen molecules resulted in reduced secretion into fibroblast medium, we measured the ratios of type I to type III collagen chains in fibroblast medium after a 16-h incubation with [<sup>3</sup>H]proline. Type I collagen/type III collagen ratios were 3.14 (II-5) and 3.85 (III-6) for OI cell strains compared with 3.58 for control. Quantitation of collagen production by bacterial collagenase assay (29) (Table II) demonstrated that for OI cells, 22% (II-5) and 33% (III-6) of incorporated [<sup>3</sup>H]proline was in collagenous protein compared with 25% for control. Finally, to determine whether the mutation in  $\text{pro}\alpha 2(I)$  altered the rate of production of  $\text{pro}\alpha 2(I)$  chains, ratios of newly synthesized  $\text{pro}\alpha 1(I)$  to  $\text{pro}\alpha 2(I)$  chains were determined. The ratio of  $\text{pro}\alpha 1(I)$  to  $\text{pro}\alpha 2(I)$  was 2.12 for one OI cell strain (II-5) and was 2.02 for control.

## Discussion

Cultured dermal fibroblasts from two affected members of a large family in which OI type IV is linked to the  $\text{pro}\alpha 2(I)$  gene

synthesize two populations of  $\text{pro}\alpha 2(I)$  chains. One population is normal and is present in both medium and cell layer samples. A second population, found only within the cells, is shortened by about 10 amino acid residues and the CNBr peptide fragment containing the apparent deletion has a more acidic isoelectric point than the normal peptide. The effect of the mutation is to cause increased posttranslational modification of the amino-terminal half of some intracellular  $\alpha 1(I)$  chains, presumably those  $\alpha 1(I)$  chains that are incorporated into molecules that also contain the shortened  $\alpha 2(I)$  chain. The melting temperature of type I collagen molecules that contain the mutant chain is 35°C, as compared with 42°C for type I collagen molecules that contain normally migrating chains; the abnormal molecules are either selectively retained within fibroblasts or very rapidly degraded after secretion under standard labeling conditions in tissue culture. The presence of the shortened  $\alpha 2(I)$  chains in the OI cells confirms that in this family, the (+) allele at the polymorphic EcoRI site in the  $\text{pro}\alpha 2(I)$  gene on chromosome 7 contains a mutation that is responsible for the OI type IV phenotype.

Presence of overmodified  $\text{pro}\alpha 1(I)$  chains in molecules with a mutation in  $\alpha 2(I)$  requires explanation. The three most likely possibilities are: first, that the patients are genetic compounds, heterozygous for a mutation in both  $\text{pro}\alpha 1(I)$  and  $\text{pro}\alpha 2(I)$ ; second, that there is a generalized abnormality in the activity or availability of collagen-modifying enzymes (prolyl and lysyl hydroxylases, and glucosyl and galactosyl transferases); and third, that abnormal triple helical formation resulting from a mutation in a single mutant chain can cause overmodification of wild-type  $\text{pro}\alpha$  chains that are contained within the same molecule. We think that genetic and biochemical evidence favors the third explanation. The first explanation, that the affected family members are heterozygotes for mutations in both  $\text{pro}\alpha 1(I)$  and  $\text{pro}\alpha 2(I)$ , can be discarded because the OI type IV phenotype in this pedigree is inherited in an autosomal dominant fashion and the genes for  $\text{pro}\alpha 1(I)$  and  $\text{pro}\alpha 2(I)$  are unlinked (38, 39). The second possibility, that there is a generalized, abnormal increase in the activity of one or more of the modifying enzymes is untenable because (a) only some  $\text{pro}\alpha 1(I)$  chains are overmodified, (b) overmodification occurs only in the amino-terminal half of the triple helical domain in affected chains, and (c) type III collagen, which is modified by the same enzymes, is not overmodified. The most likely explanation for overmodified  $\text{pro}\alpha 1(I)$  chains is that the mutation in  $\alpha 2(I)$  disrupts triple helix formation. A review of the requirements for normal triple helical structure provides understanding of how a mutation in one chain

**Table II.** Incorporation of [<sup>3</sup>H]proline into Total and Collagenous Protein by Control and Osteogenesis Imperfecta Cells in Culture

Cell strain		Total protein	Collagen	Cpm in collagen	Collagen in medium
		<i>cpm</i> × 10 <sup>-3</sup> /2.5 × 10 <sup>5</sup> cells	<i>cpm</i> × 10 <sup>-3</sup> /2.5 × 10 <sup>5</sup> cells	%	%
Control	Medium	158.0	69.0	43.6	
	Cells	154.7	7.7	4.9	
	Total	312.7	76.7	24.5	90
III-6	Medium	142.7	53.3	37.3	
	Cells	115.8	4.9	4.2	
	Total	258.5	58.2	22.5	91.5
II-5	Medium	153.2	66.4	37.3	
	Cells	106.7	17.2	16.0	
	Total	259.9	83.6	32.2	80

may produce overmodification of the mutant and wild-type chains within the same molecule, as well as decreased triple helical stability. Triple helix formation depends on having glycol residues in every third position (Gly-X-Y)<sub>n</sub> and the structure is stabilized by an abundance of Y-positioned hydroxyprolyl residues. Hydroxyproline within the triple helix is formed by enzymatic hydroxylation of proline, which together with hydroxylation of certain lysyl residues, occurs as a modification of newly translated pro $\alpha$  chains. Triple helix formation is initiated at the carboxyl end and proceeds toward the amino-terminal end, and once a stable triple helix is formed, further modification is inhibited (40). We think that the mutation in the middle of the triple helical domain of pro $\alpha$ 2(I) in cells from this family disrupts triple helical propagation and the unwound chains amino-terminal to the mutation are available for additional modifications.

We do not know whether the deletion in  $\alpha$ 2(I) reported here disrupts the (Gly-X-Y)<sub>n</sub> sequence. Data from another OI cell strain demonstrate that decreased triple helix stability and increased posttranslational modification can result from a mutation in the chains of type I collagen that disrupts the triplet sequence. In that cell strain, which is from an infant with lethal OI type II, a single base change produces a Gly to Cys substitution in  $\alpha$ 1(I)CB6, which results in a lowered melting temperature in abnormal molecules as well as overmodification of all the constituent chains (12, 17). However, evidence from another lethal OI cell strain suggests that a deletion within the triple helical domain may produce similar effects even if the (Gly-X-Y)<sub>n</sub> sequence is preserved. That cell strain was heterozygous for an intron-to-intron deletion of three exons, with preservation of (Gly-X-Y)<sub>n</sub> in the shortened chain. The melting temperature of molecules that contained the abnormal allelic product was lower than normal and in those molecules there was overmodification of the amino-terminal region of  $\alpha$ 1(I) (7, 8, 10, 11; Bonadio, J. F., and P. H. Byers, unpublished observations).

Failure to detect overmodified pro $\alpha$ 2(I) chains in the cell strains described in the present report may reflect the fact that pro $\alpha$ 2(I) is normally modified to a greater extent than pro $\alpha$ 1(I) (16), so that there may be few additional sites in the amino-terminal half of  $\alpha$ 2(I) chains available for further modification. Further, failure to detect the shortened unmodified pro $\alpha$ 2(I) probably reflects limitations of resolution (Fig. 2 B). This OI cell strain provides additional support for the concept that overmodification of chains of type I collagen is common in cells from patients with certain forms of OI, that the extent of overmodification helps to locate a domain that contains a mutation, and that the abnormal structure contributes to the pathophysiology of the disease (1, 12, 13, 15, 25).

OI type IV is a mild to moderately severe phenotype characterized by white or grey sclerae, bone fragility, moderate short stature with frequent deformity, a high incidence of dentinogenesis imperfecta, and autosomal dominant inheritance. Based on the biochemical findings in this report, which confirm genetic linkage of OI type IV to the pro $\alpha$ 2(I) locus in this family, and on linkage studies on other OI type IV families (27, 28), it now appears that the clinical distinction between OI type I and OI type IV is the result of different mutations in the genes of type I collagen, which in turn have differing effects on the amount and quality of bone matrix. In classic OI type I, there is simply a decrease in the amount of type I collagen produced by dermal fibroblasts (1, 4, 6) (though the molecular causes will undoubtedly prove to be heterogeneous), but in most cases of OI type IV there is a subpopulation of type I collagen molecules with altered

triple helical structure and increased posttranslational modification (41), probably due to subtle mutations in pro $\alpha$ 2(I). It is unclear whether ineffective bone mineralization is the result of overmodification, as has been suggested (42, 43), or whether it is due to the mutation itself. We are uncertain whether molecules that contain the mutant  $\alpha$ 2(I) chains from the present family are secreted by osteoblasts or are incorporated into bony matrix. An analysis of type I collagen from an affected family member's bone would be very useful if such tissue becomes available for study. That OI fibroblasts from this family do not accumulate significant amounts of abnormal molecules in the culture medium even during very long labeling periods (data not shown) may account for the mild clinical phenotype. The OI cells also, surprisingly, secrete approximately normal amounts of type I collagen. Though we do not yet understand the mechanism for this, it may be another factor by which the severity of the clinical phenotype is modified.

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