A Tripeptide Deletion in the Triple-helical Domain of the Proα1(I) Chain of Type I Procollagen in a Patient with Lethal Osteogenesis Imperfecta Does Not Alter Cleavage of the Molecule by N-Proteinase*

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Dermal fibroblasts from a fetus with perinatal lethal osteogenesis imperfecta synthesized normal and abnormal type I procollagen molecules. The abnormal molecules contained one or two proα1(I) chains in which glycine, alanine, and hydroxyproline at positions 874, 875, and 876 in the triple-helical region were deleted as the result of a 9-base pair genomic deletion. Molecules that contained abnormal chains were overmodified from the site of the deletion toward the amino-terminal region of the molecule. Secretion of the overmodified molecules was impaired. The thermal stability of molecules containing abnormal chains was lower than that of normally modified molecules. After cleavage of molecules with vertebrate collagenase, the temperature of thermal denaturation of the overmodified fragments was greater than that of the fragments from the normal molecules. The rates of cleavage of the normal and the abnormal molecules by N-proteinase were indistinguishable. Our findings suggest that the tripeptide deletion introduces a shift in the phase of the chains in the triple helix. This structural change is propagated from the site of the deletion toward the amino terminus of the molecule, but the subsequent alteration in the structure of the N-proteinase cleavage site is not sufficient to cause a decrease in the rate of cleavage by the enzyme.

More than 70 mutations in the two genes that encode the proα1(I) and proα2(I) chains (COL1A1 and COL1A2, respectively) of type I collagen have been identified as the molecular cause of different forms of osteogenesis imperfecta (OI) (1, 2). Mutations causing the perinatal lethal form of OI (OI type II) include genomic deletions (3, 4), an insertion in COL1A1 (5), splicing errors (6, 7), and point mutations that lead to the substitution of single glycine residues within the Gly-X-Y repeat unit characteristic of the collagen triple helix (1, 2). Most of the mutations interfere with the normal assembly of a stable triple helix, delay secretion, and cause increased posttranslational modification (lysyl and prolyl hydroxylation and hydroxylsyl glycosylation) of all chains amino-terminal to the site of the alteration in the molecule (7–9). An additional feature of molecules that contain chains with deletions or amino acid substitutions for glycine is that they are resistant or partially resistant to cleavage by N-proteinase (10, 11). Type I procollagen N-proteinase removes the amino-propeptide domain from the type I procollagen molecule during the normal processing of procollagen to collagen (12, 13). Previous studies have demonstrated that N-proteinase will not cleave heat-denatured procollagen and is a substrate conformation-dependent proteinase (14). To explain how an amino acid substitution in the triple-helical region of the type I procollagen molecule results in slower processing by N-proteinase, it has been proposed that a conformational change, such as a tripeptide shift in the phase of the chains in the helix (15), is propagated from the site of the substitution toward the amino terminus and thereby disrupts the N-proteinase cleavage site (10, 11).

We have characterized a mutation in a fetus with OI type II that results in the deletion of a tripeptide: glycine, alanine, and proline (normally hydroxylated to hydroxyproline) from that manner subsequently) at positions 874, 875, and 876 of the triple helix of the proα1(I) chain (residue 1 is the first glycine of the major triple helix). We have shown that an altered structure is propagated from the site of this deletion toward the amino-terminal region of abnormal molecules but that the rates of cleavage of the normal and abnormal molecules by N-proteinase are indistinguishable. These findings demonstrate that a tripeptide shift in the phase of the chains in abnormal molecules does not alter the N-proteinase cleavage site in such a way as to cause a decrease in the rate at which the enzyme cleaves the amino-terminal propeptide from those molecules.

MATERIALS AND METHODS

Clinical History—The infant was the first child born to a 36-year-old mother and 32-year-old father. Neither parent, none of the mother's four older children, nor any of the father's four older children (all with previous spouses) had evidence of osteogenesis imperfecta. This pregnancy was unremarkable by history, and fetal activity was

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felt to be normal. There was a question of polyhydramnios detected by ultrasound. Because of frank breech presentation at the onset of labor at 32 weeks gestation, delivery was by cesarean section. The infant weighed 1,500 g, was 33 cm long, and had a head circumference of 31 cm. Apgar scores at 1 and 5 min were 1 and 1, respectively, and there was a diagnosis of respiratory failure at delivery. The head was soft with no palpable cranium, the chest was small, florid cyanosis of the extremities was short and bowed. Radiographs were consistent with the diagnosis of the perinatal lethal form of osteogenesis imperfecta (OI type II). Chromosomes were normal male.

Preparation and Electrophoretic Analysis of Procollagens and Collagen—Flibroblast cell strains were established from explants of skin from the fetus with OI type II (87-126). Cells from unrelated healthy subjects served as controls. Cell cultures were maintained under standard conditions in Dulbecco-Vogt modified Eagle’s medium (Irvine Scientific) as described previously (16). Labeling of proteins with 2,3,4,5-[^3H]proline (101 Ci/mmol, Amersham Corp.), harvesting of the medium and cell layer proteins, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), cleavage of proteins with cyanogen bromide (CNBr) in gels, and two-dimensional analysis of the resultant peptides of type I collagen were carried out as described previously (8, 16). Vertebrate collagenase fragments of the α(I) and α(2) chains were prepared from pepin-digested [3H]proline labeled procollagens by digestion with fibroblast collagenase for 16 h at 20 °C (17). The collagenase was provided by Dr. Eugene Bauer, Stanford University. Vertebrate collagenase cleaves type I collagen between amino acids 775 and 776 of the triple helix, generating the aminoterminal A fragment and the carboxyterminal B fragment.

Preparation and Purification of Procollagen—[^3C]-Labeled type I procollagen was purified from the culture medium of normal and proband fetal fibroblasts (passages 7-10) using the methods described previously (18). In brief, fibroblasts were grown to confluence and incubated in Dulbecco’s modified Eagle’s medium supplemented with 1 μCi/ml of a mixture of uniformly labeled [14C]L-lysine acids, 25 μg/ml ascorbic acid, and no serum. Medium proteins were precipitated by ammonium sulfate, and the type I procollagen was chromatographed on two consecutive columns of DEAE-cellulose (19, 20). The procollagen was concentrated by ultrafiltration and stored at −20 °C in stock solution consisting of 1.5 M Tris buffer (pH 7.4, 20 °C) containing 0.4 M NaCl and 0.01% NaN3. Procollagen concentration was determined by a colorimetric hydroxyproline procedure (21), assuming 10.1% hydroxyproline by weight procollagen (19). The preparation had 800 units/ml of activity where 1 unit = 1 μg of proal(I) chains in the sample were not resolved sufficiently by SDS-PAGE.

RESULTS

Synthesis of Abnormal Molecules and Localization of an Abnormality in α(I)CB6 of Type I Procollagen—Cells from the infant with OI produced normal molecules and molecules that contained α chains that were delayed in electrophoretic mobility. The chains in the abnormal molecules were preferentially retained by the cells (Fig. 1A). The altered electrophoretic mobility of the chains synthesized by the cells from the fetus was the result of overmodification of chains of type I procollagen along their full-length (Fig. 1B).

A 9-Base Pair Genomic Deletion Leads to an In-frame Deletion of a Tripeptide—The cDNA clones encoding the α(I)CB6 region were either of normal sequence or contained a 9-bp deletion (either 5′ GTGCCCTG 3′ or 5′ GTGCCCTGTG 3′) which would result in the deletion of amino acid residues Gly326, Ala327, and Hyp328 of the proα(I) triple-helical domain (Fig. 2). This deletion left the frame of the remaining coding sequence intact. To confirm that the deletion did not arise during the preparation of the cDNA or during the amplification process, genomic DNA was isolated from the OI cell strain and from a control cell strain that spanned exon 44. The resultant 225-bp product was digested with the restriction endonuclease BanI, and the digestion products were analyzed on a 6% PAGE gel.
The lack of an additional sequence change in the COL1A2 region of the gene corresponding to the CB6 region of proc1(I) confirmed that the observed overmodification resulted from the Gly-Ala-Hyp tripeptide deletion.

**Rotary Shadowing Electron Microscopy**—The type I procollagens purified from the medium of the OI and normal cells in culture were examined by rotary shadowing electron microscopy. Analysis of 200 molecules from each sample showed that the OI molecules were indistinguishable from those in the control samples in that they were thread-like, approximately 300 nm in length, and did not have kinks as has been observed in type I procollagen molecules containing substitution of cysteine for glycine residues (15, 45).

The Melting Temperature of the Overmodified Collagen Is Decreased, whereas the Overmodified Collagenase A Fragments Have an Increased Thermal Stability—The overmodified type I collagen molecules isolated from the medium and cell layers of the OI cell strain had a Tm of 41 °C, whereas normal molecules synthesized by control cells and by the OI cells had a Tm of 42 °C (Fig. 3). Following cleavage with fibroblast collagenase of the collagens isolated from the medium and cell layer of the OI fibroblasts, the overmodified collagenase A fragments melted at 38 °C whereas the normally modified A fragments and the A fragments from the control molecules melted at 36 °C (Fig. 4). The rate of cleavage by collagenase of the abnormal molecules was not slower than that of the normal molecules.

The vertebrate collagenase A fragments of type III collagen migrate in gels to approximately the same position as overmodified 1(I) chains and have a thermal stability slightly above that of normally modified 1(I) chains (32). To confirm that we were observing the thermal denaturation of the normally modified and the overmodified A fragments of type I collagen, the thermal stability assay was repeated using a preparation of purified type I procollagen that lacked type III procollagen. The thermal denaturation of this material was as obtained for the unpurified material (results not shown).

**Cleavage of the Abnormal Molecules by N-Proteinase**—In experiments that examined the consequences of the deletion for cleavage of the procollagen by N-proteinase, purified type I procollagen was incubated with partially purified N-proteinase in vitro under conditions previously shown to detect changes in activity of the enzyme toward the substrate (10, 45). The abnormal procollagen molecules containing one or more normal sequences did not allow significant cleavage, in contrast to the normal procollagen molecules.

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**Fig. 1.** Two-dimensional CNBr peptide analysis of 1 chains. Panel A, pepsin-treated procollagens, yielding 1 chains, separated under nonreducing conditions by SDS-PAGE in a 5% gel. The arrows indicate the populations of overmodified 1(I) chains in the medium and cell layers of the OI type II cell strain (OI) but not seen in the control (C). Panel B, the section of the gel represented in panel A was treated with CNBr, and the peptides were separated by SDS-PAGE in 12.5% gel. The overmodified 1(I) chains from the OI cell strain (arrows) yielded peptides that all migrated slower than those peptides from the normally migrating 1(I) chains. The CNBr peptides from the collagens of the control cell strain migrated as single discrete spots. The line diagram represents the positions of the CB peptides within the 1(I) chain; the vertical bars are the positions of the methionyl residues.

**Fig. 2.** Sequence of the 1(I) CB6 mutation. Panel A, cDNA sequencing gels showing the normal and the mutant COL1A1 alleles. The arrow marks the site of the mutation. The mutation was a 9-bp genomic deletion in exon 44 (either 5' GGTGCCCTG 3' or 5' GGTGCCCTCG 3') which would result in the deletion of amino acid residues Gly184, Ala185, and Pro186 of the proc1(I) triple-helical domain. Panel B, DNA and protein sequences surrounding the mutation site. The two possible positions of the deletion are underlined. The first glycine of the major triple helix is designated as residue 1.

**Fig. 3.** Thermal stability of type I collagen molecules. Peptis-digested samples were gradually heated, aliquots removed at the temperatures indicated and treated with trypsin. A and B represent the collagens isolated from the medium and cells, respectively, of control cells; C and D represent the collagens present in the medium and cells, respectively, of the OI cells. The abnormal molecules that contain overmodified chains in the medium and cell layer of the OI cells melted at 41 °C; the control collagen and the normally modified collagen synthesized by the OI cells melted at 42 °C.
two abnormal proc(1) chains were posttranslationally overmodified and migrated slowly in SDS gels. The difference in migration of the proc chains was not sufficient to allow laser densitometry of the normally migrating and slowly migrating chains separately, so the normally migrating and slowly migrating proc chains were analyzed together. The rates of cleavage of proc(1) chains in control samples and of the normally migrating and slowly migrating proc(1) chains in the OI samples by N-proteinase were indistinguishable from each other using the methods described here (Fig. 5A). At 50 units/ml of N-proteinase instead of the usual 10 units/ml of enzyme, the rates of cleavage of control procollagen and of normal and abnormal procollagens synthesized by the OI cells were again indistinguishable from each other (inset in Fig. 5B).

DISCUSSION

Although considerable information is available about the spectrum of mutations that cause different forms of OI and it is possible, to some extent, to predict the severity of the phenotype from the nature of the causative mutation, little is known about the ways in which mutations alter the structure and properties of the collagen molecule.

Most of what is known about the effects of mutations in collagen genes on the stability of the triple helix is the result of studies of substitutions of other amino acids for glycine, the invariant occupant of every 3rd residue position in the triple helix of each of the three chains. Substitutions by residues with bulkier side chains might be expected to introduce structural changes at the site of the substitution that delay helix propagation (from carboxyl to amino terminus) or to modify the structure of the triple helix and, thereby, result in overmodification of the chains amino-terminal to the site of the substitution (because the modifying enzymes recognize the free chains or the abnormal structure). Two models have been proposed to explain how a substitution for glycine could delay helix formation. Traub and Steinmann (33) proposed that where a cysteine is substituted for a glycine, a bulge in the GIy-X-Y repeat unit is overmodified, but it seems likely that specific interactions between the -X and -Y residues in adjacent chains are important in determining the rate of helix formation or the structure of the triple helix.

Fig. 4. Thermal stability of the A fragments of α1(I) and α2(I) generated by digestion of type I collagen by vertebrate collagenase. Samples were heated to the temperatures indicated, aliquots were treated with trypsin, and the resistant chains were separated on a 7% SDS-PAGE gels. A and B represent the collagenous peptides generated from collagen isolated from the medium and cell layer, respectively, of control cells; C and D are collagenous peptides generated from the medium and and cell layer, respectively, of the OI cells. The overmodified A fragments from the medium and cell layer of the OI cells melted at 38 °C; the control A fragments and the normally modified A fragments synthesized by the OI cells melted at 36 °C.

Fig. 5. N-Proteinase cleavage of control and OI type I procollagen. The type I procollagens purified from the medium of OI, and control dermal fibroblasts in culture were incubated with N-proteinase at 34 °C in a reaction system that comprised 10 μg/ml procollagen and 100 units/ml enzyme. At the times indicated the reaction was stopped by the addition of SDS sample buffer, the products were separated by SDS-PAGE in a 5% gel and the collagens visualized by fluorography. A, fluorograms showing conversion of proc(1) and proc(2) chains to pCa(1) and pCa(2) chains, respectively; 24-E denotes samples incubated for 24 h at 34 °C in the absence of N-proteinase. B, laser densitometry of fluorograms confirmed that the rate of cleavage of the OI procollagen (Δ) was indistinguishable from that in control samples (●). The inset shows the rate of cleavage of normal and OI procollagens by 50 units/ml N-proteinase at 34 °C.
The thermal stability of the overmodified collagen containing the tripeptide deletion was reduced by 1 °C. The discrepancy between our findings and those reported by Hawkins et al. (34), who identified a similar deletion in one of the adjacent Gly-Ala-Hyp tripeptides, is unclear but may reflect minor differences in the methods used to measure thermal stability. The denaturation of type I collagen is a highly cooperative process, and no unfolding intermediates are observed (35, 36). Microcalorimetry measurements of the triple helix over a wide range of temperatures shows that blocks of the triple helix "micro-unfold" in the pre-denaturation range of temperatures (36, 37). Denaturation of the triple helix is, therefore, envisaged as a cooperative unfolding of adjacent blocks of residues until, at the denaturation temperature, a critical length of the molecule is unfolded sufficiently to prevent refolding of the chains (38). One explanation for why the deletion of the Gly-Ala-Hyp tripeptide decreases the thermal stability of the triple helix is that the region of 874–876 is particularly stable. Gly-Ala-Hyp tripeptides occur in clusters along the a1(I) chain (39). These clusters can form stable molecules by fibroblast collagenase was not markedly delayed; the propagation of a conformational change from the site of an amino acid substitution toward the amino terminus has been proposed to explain how amino acid substitutions in the triple-helical region of the type I procollagen molecule could result in slower processing of the molecule by N-proteinase. In the model proposed by Vogel et al. (15), it was suggested that the tripeptide shift introduced to accommodate the cysteine substitution altered the putative hairpin structure formed by each of the three pro-collagen chains in the region containing the N-proteinase cleavage site and that the altered conformation was responsible for the slower cleavage by N-proteinase. However, we have shown that N-proteinase readily cleaves molecules containing a tripeptide deletion. This finding offers direct evidence that a tripeptide shift that is propagated toward the amino terminus does not alter the N-proteinase cleavage site in such a way as to reduce the rate of cleavage by the enzyme. Therefore, it seems likely that even if a tripeptide shift were introduced at the site of a substitution for glycine, it is not sufficient to explain the decreased rate of cleavage by N-proteinase. Lightfoot et al. (45) have excluded the possibility that the site of a substitution for glycine sequesters enzyme molecules and thereby acts as a competitive inhibitor. Instead other alterations in structure such as a change in chain order, change in helix pitch, or modifications of residue in or near the cleavage site might be invoked to explain the abnormal kinetics of cleavage of those molecules containing substitutions for glycine.

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