Biochem. J. (1991) 279, 747–752 (Printed in Great Britain)

Substitution of cysteine for glycine-α1-691 in the proα1(I) chain of type I procollagen in a proband with lethal osteogenesis imperfecta destabilizes the triple helix at a site *C*-terminal to the substitution

Beat STEINMANN,* Andrea WESTERHAUSEN,† Constantinos D. CONSTANTINOU,† Andrea SUPERTI-FURGA* and Darwin J. PROCKOP†‡

*Division of Metabolism, Department of Pediatrics, University of Zürich, CH-8032 Zürich, Switzerland, and †Department of Biochemistry and Molecular Biology, Jefferson Institute of Molecular Biology, Jefferson Medical College, Thomas Jefferson University, Philadelphia, PA 19107, U.S.A.

Skin fibroblasts from a proband with lethal osteogenesis imperfecta synthesized a type I procollagen containing a cysteine residue in the α1(I) helical domain. Assay of thermal stability of the triple helix by proteinase digestion demonstrated a decreased temperature for thermal unfolding of the protein. Of special importance was the observation that assays of thermal stability by proteinase digestion revealed two bands present in a 2:1 ratio of about 140 and 70 kDa; the 140 kDa band was reducible to a 70 kDa band. Further analysis of the fragments demonstrated that the cysteine mutation produced a local unfolding of the triple helix around residue 700 and apparently exposed the arginine residue at position 704 in both the α1(I) and α2(I) chains. Analysis of cDNAs and genomic DNAs demonstrated a single-base mutation that changed the GGT codon for glycine-691 of the α1(I) chain to a TGT codon for cysteine. The mutation was not found in DNA from either of the proband's parents. Since the proteinase assay of helical stability generated a fragment of 700 residues that retained disulphide-bonded cysteine residues at α1-691, the results provide one of the first indications that glycine substitutions in type I procollagen can alter the conformation of the triple helix at a site that is *C*-terminal to the site of the substitution.

INTRODUCTION

Over 80 mutations that change the primary structure of procollagen (see Prockop, 1990; Byers, 1990; Kuivaniemi *et al.*, 1991) have been found in probands with osteogenesis imperfecta, a heritable disorder characterized by brittleness of bone. With a few exceptions, most of the mutations cause synthesis of a structurally abnormal but partially functioning proα1(I) or proα2(I) chain of type I procollagen. The most common mutations found to date have been a series of single-base mutations that substitute amino acid residues with bulkier side chains for the glycine residues (Steinmann *et al.*, 1984; Prockop, 1990; Byers, 1990; Kuivaniemi *et al.*, 1991) that appear as every third residue in the repeating Gly-Xaa-Yaa- sequence of the triple-helical domain of type I procollagen (see Piez, 1984). Some of the glycine substitutions that cause lethal variants of osteogenesis imperfecta prevent folding of the protein into a triple helix and cause degradation of monomers containing normal and abnormal proα1(I) and proα2(I) chains through a process referred to as procollagen suicide (Williams & Prockop, 1983; Prockop, 1990). Other glycine substitutions that cause lethal variants of osteogenesis imperfecta have little, if any, effect on the thermal stability of the triple helix. Instead, they appear to allow folding but alter the conformation of the molecule sufficiently to affect self-assembly into collagen fibrils. A mutation substituting cysteine for glycine-α1-748 produced a flexible kink in the triple helix detectable by rotary-shadowing electron microscopy (Vogel *et al.*, 1987, 1988). Molecules containing the flexible kink were then found to form co-polymers with normal collagen synthesized by the same fibroblasts (Prockop *et al.*, 1989; Kadler *et al.*, 1991). Formation of the co-polymers distorted the morphology of the fibrils, decreased the rate of fibril formation and decreased the net amount of collagen incorporated into fibrils at steady-state equilibrium. To date, however, it is not apparent why some glycine substitutions markedly alter the functional properties of procollagen whereas others do not. Also, there is no simple explanation as to why most of the reported glycine substitutions cause lethal variants of osteogenesis imperfecta whereas some glycine substitutions produce much milder phenotypes.

Here we demonstrate that a proband with a lethal variant of osteogenesis imperfecta had a single-base substitution that converted the codon for glycine-α1-691 in one allele for proα1(I) chains of type I procollagen into a codon for cysteine. The presence of the glycine substitution made the triple helix susceptible to cleavage by trypsin. Surprisingly, however, the cleavage occurred *C*-terminal to the mutation even though the collagen triple helix folds from the *C*-terminal to the *N*-terminus (Bächinger *et al.*, 1980; Engel, 1987; Prockop, 1990; Engel & Prockop, 1991). Also, the intermediate generated by the cleavage by trypsin was more stable to further proteinase digestion than the larger A fragment of 775 residues generated by cleavage of normal collagen with vertebrate collagenase.

MATERIALS AND METHODS

The proband

The proband (M. G., 24 April 1987) was the first child born to young healthy unrelated Swiss parents. The family history was unremarkable with regard to connective-tissue disorders. After an uneventful pregnancy of 39½ weeks, the baby was born from

Abbreviation used: ASO, allele-specific oligonucleotide.

‡ To whom correspondence should be addressed.

Table 1. Primers used to generate PCR products

Primers were used in combinations indicated in the text. Underlinings indicate sites for restriction with *Eco*RI or *Bam*HI that are convenient for cloning the PCR products.

Template	Target sequence: Sense	Target sequence: Antisense	Primer sequence
cDNA		α1-358–364	CTGGAATTCCTGGTGCCAAGGGTCTGACTG
cDNA	α1-563–570		TTAGGATCCGCATCACCTCTGTCACCCTTA
cDNA		α1-549–555	CCTGGAATTCAGGGAATGCCTGGTGAACGT
cDNA	α1-723–730*		CACGGATCCCTTTGCCGCCTTCTTTGCCAG
cDNA		α1-674–681	GGGGGAATTCCATTGGTAATGTTGGTGCTC
cDNA	α1-844–851		AAAAGGATCCAACCGTCTCGTCCAGGGGAA
gDNA		α1-Intron 35	CAGACGCATCTCTCCAATCT
gDNA	α1-Exon 40*		CACGGATCCCTTTGCCGCCTTCTTTGCCAG
cDNA		α2-396–402	CGGATCCAGCAAGAGGAGAGCCTGGCAACATT
cDNA	α2-549–555		GAGGAATTCTCTCCTGGGAGTCCACT
cDNA		α2-516–522	GAGGATCCATTGGAAGCCGAGGTCCTTC
cDNA	α2-724–730		CCTCGAATTCCACGAAGCCCTTCTTTCCCA

* Identical primers used for both cDNA and genomic DNA (gDNA) templates.

a vortex position 9 h after rupture of the membranes. Her weight was 1950 g, length about 40 cm and head circumference 32 cm. The placenta weighed 270 g. She presented with short bowed extremities, dark blue sclerae and a soft skull resembling a 'balloon filled with water', which suggested breech presentation to the obstetrician. She was intubated, ventilated and transferred to the University Children's Hospital, Zürich, Switzerland, where a diagnosis of osteogenesis imperfecta type IIA was made on the basis of the clinical and radiological findings. A few minutes after extubation, the baby girl died from respiratory insufficiency. Autopsy revealed lung hypoplasia with inter- and intra-alveolar haemorrhage as the cause of death.

Cell culture and biochemical studies

Skin fibroblasts were obtained from the patient, her parents and normal controls with informed consent, and were grown under standard conditions (Steinmann *et al.*, 1984). Radiolabelled procollagens were isolated from cell cultures (passages 2–8) incubated with ascorbic acid, β-aminopropionitrile, [^{3}H]proline and [^{3}H]glycine at 37 °C or 30 °C (Steinmann *et al.*, 1984; Superti-Furga & Steinmann, 1988). Triple-helical collagens were prepared by digestion of procollagens with pepsin (Steinmann *et al.*, 1984). The thermal stability of pepsin-purified collagens was determined by trypsin digestion as described by Bruckner & Prockop (1981) and modified by Superti-Furga & Steinmann (1988). Briefly, samples of collagen dissolved in 0.4 M-NaCl/0.1 M-Tris/HCl buffer, pH 7.4, were heated gradually to different temperatures, cooled quickly to 20 °C, digested for 2 min with trypsin (tosylphenylalanylchloromethane-treated, Worthington; 20 μg/ml) and immediately frozen and freeze-dried. The samples were redissolved in hot sample buffer and analysed by SDS/PAGE. Cleavage of pepsin-purified collagens with vertebrate collagenase (generously given by Dr. B. Adelmann, Max-Planck-Institut, Münich, Germany) was performed in 50 mM-Tris/HCl buffer, pH 7.4, containing 10 mM-$CaCl_2$ and 2 mM-phenylmethanesulphonyl fluoride at room temperature for 16 h. Preparations were analysed by SDS/PAGE using the buffer system of Laemmli (1970) containing 0.5 mM-urea (Steinmann *et al.*, 1984). Two-dimensional mapping of CNBr-cleavage peptides of collagen chains (cleavage time 90 min) was performed as described by Steinmann *et al.* (1984). After electrophoresis, the radiolabelled collagens were detected by autoradiofluorography (Laskey & Mills, 1975).

Extraction of skin collagen

The skin was kept frozen, then dissected free of adhering adipose tissue, finely minced and extracted with pepsin (100 μg/mg of skin per ml of 0.5 M-acetic acid) for 20 h at 4 °C. The clarified extract was freeze-dried, then resuspended in sample buffer, and the collagens were analysed by SDS/PAGE. The gels were stained with Coomassie Brilliant Blue R-250.

Electron microscopy of skin

Electron microscopy of the skin was performed as described by Steinmann *et al.* (1984).

Definition of the mutation in mRNA-derived cDNA and genomic DNA

To prepare cDNA for use as a template for the PCR (Saiki *et al.*, 1985), total RNA was extracted by a procedure involving extraction with guanidinium isothiocyanate and centrifugation on a CsCl gradient (Maniatis *et al.*, 1982). The RNA was used to synthesize double-stranded DNA as described by Gubler & Hoffman (1983). To isolate genomic DNA templates for the PCR, DNA was extracted from 175 cm^2 flasks of skin fibroblasts with proteinase K and SDS (Maniatis *et al.*, 1982).

For experiments with cDNA as a template for the PCR, three sets of oligonucleotide primers complementary to the cDNA sequence of the proα1(I) chain and two sets complementary to the cDNA sequence of the proα2(I) chain were used (Table 1). The oligonucleotides were 18- to 32-mers that consisted of complementary sequence and additional sequences for restriction sites convenient for cloning. For analysis of the proα1(I) chain, primer set I spanned codons for amino acid residues 358–364 and 563–570 (Bernard *et al.*, 1983*b*; Tromp *et al.*, 1988). Primer set II spanned codons for amino acid residues 549–555 and 723–730. Primer set III spanned codons for amino acid residues 674–681 and 844–851. For analysis of the proα2(I) chain, primer set I spanned codons for amino acid residues 396–402 and 549–555 (Bernard *et al.*, 1983*a*; Kuivaniemi *et al.*, 1988). Primer set II spanned codons for amino acid residues 516–522 and 724–730. The PCR products were cloned into the filamentous bacteriophage M13 mp18 or M13 mp19, and the clones were sequenced with the dideoxynucleotide chain-termination method (Sanger *et al.*, 1977).

To use genomic DNA as template for the PCR, one primer

was complementary to the 3′ region of intron 35 of the proα1(I) gene and the other was complementary to codons for amino acid residues 723–730 in exon 40 (Bernard *et al.*, 1983*b*; Tromp *et al.*, 1988). The PCR products were dot-blotted on to nylon filters and then hybridized with allele-specific oligonucleotides (Studencki & Wallace, 1984). The oligonucleotides contained either the normal sequence around the codon for glycine-α1-691 or the mutated sequence. The oligonucleotide for the normal sequence was -GGCAGCGCTGGTCCCCCTGGT-. The oligonucleotide for the mutated sequence had the same sequence except that a central G (underlined) was converted into a T. The blots were pre-hybridized for 2 h in 6 × SSC (1 × SSC is 0.15 M-NaCl/0.015 M-sodium citrate buffer, pH 6.8), 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll 400/0.02% polyvinylpyrrolidone/0.02% BSA), 0.5% SDS and 0.25 mg of denatured salmon sperm DNA/ml. Hybridization was performed at 60 °C for 4 h after adding γ-^{32}P-labelled oligonucleotides to the same solution. The filters were washed four times in 2 × SSC/0.1% SDS at room temperature for 15 min. The sample probed with the normal sequence was then washed in 6 × SSC at 72 °C for 3 min. The sample probed with the mutated sequence was washed in the same solution at 70 °C for 3 min.

RESULTS

Analysis of procollagen synthesized by the proband's fibroblasts

Procollagens were cleaved to collagens with pepsin and the collagens were analysed by SDS/PAGE. As indicated in Fig. 1, a slowly moving band of disulphide-linked α1′(I) chain was seen. The protein in the band was identified by two-dimensional gel electrophoresis: when reduced in the second dimension, the band co-migrated with the α1(I) chains (Fig. 3). Furthermore, after CNBr cleavage of the collagen chains within the gel, electrophoresis in the second dimension under reducing conditions revealed a peptide pattern derived from the α1′(I)-dimer that was indistinguishable from that of the α1(I) chain (results not shown). However, when electrophoresis in the second dimension was done without reduction, the α1(I) CB7 peptide as such was absent but was present as its dimer (Fig. 1).

In order to localize further the cysteine residue within the α1(I) CB7 peptide, collagen was digested with human fibroblast collagenase. Gel electrophoresis revealed the expected A and B fragments of 75 and 25 kDa respectively, derived from the α1(I) and α2(I) chains. In addition, a 150 kDa component was detected that was converted by reduction into an A fragment of an α1(I) chain. We therefore concluded that the patient was heterozygous for a cysteine substitution that was located in the CB7 peptide region (residues 552–822) of the α1′(I) chain and *N*-terminal to residue 776, the collagenase-cleavage site (see Bernard *et al.*, 1983*a*,*b*). The fibroblasts thus produce three different species of collagen I molecules, containing two, one or no mutant α1′(I) chains (see below).

The molecules containing one or two mutant chains were overmodified as judged by the diminished electrophoretic mobility (Fig. 1), which reverted to normal when cells were incubated with 0.3 mM-αα′-bipyridyl (results not shown). Assay by brief proteinase digestion demonstrated that molecules containing one or two mutant α′1(I) chains were thermally unstable, in that they had T_m values of 40.0–40.5 °C and 38.5 °C respectively, as compared with 41 °C for normal collagen I (Fig. 2). Of special importance was the observation that the assays of thermal stability by brief proteinase digestion revealed two bands that were present in a 2:1 ratio and that were about 140 and 70 kDa. The 140 kDa band was reducible to a 70 kDa component (Fig. 3). The results therefore indicated that the mutation produced a local unfolding of the triple helix around residue 700 and this probably exposed the arginine residues at position 704 in both α1(I) and α2(I) chains (Bernard *et al.*, 1983*a*,*b*) and made them susceptible to cleavage by trypsin.

Further experiments demonstrated that incubation of the cells at 30 °C rather than at 37 °C partially reversed the untoward effects of the mutation on collagen metabolism: overmodification of collagen was less pronounced and the amount of mutant collagen secreted into the medium, as judged by the amount of α1′(I) dimers, was increased as compared with collagen III as a reference protein (Fig. 1, horizontal lanes). Similar effects of temperature were noted previously with a cysteine-for-glycine substitution (Steinmann *et al.*, 1984, 1988).

Collagen extracted from the proband's skin

Gel electrophoresis of extracted skin collagen showed overmodified α1(I) and α2(I) chains and confirmed the presence of

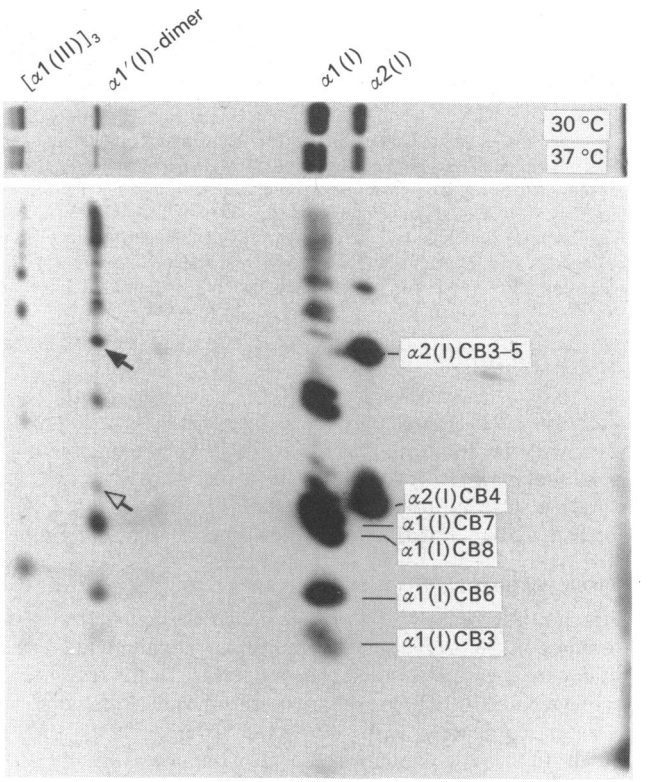

Fig. 1. Gel electrophoresis of pepsin-purified collagen secreted by the proband's fibroblasts

Collagens secreted at 37 °C or 30 °C and separated by electrophoresis on a 5% polyacrylamide gel in the first dimension are shown in the horizontal lines: at 37 °C the α1(I) and α2(I) chains are overmodified, two populations of the α1(I) chains being clearly discernible; in addition, there is a reducible α1′(I)-dimer. The collagens secreted at 30 °C, however, were less heavily overmodified, and the proportion of α1′(I)-dimer is increased. This indicates that the collagen is thermally unstable and that it can be partially rescued at lower temperatures (Superti-Furga & Steinmann, 1988; Steinmann *et al.*, 1988). Two-dimensional mapping of CNBr-cleavage peptides of collagen secreted at 37 °C was done on a 10% gel in the second dimension (lower panel). All α1(I)- and α2(I)-derived peptides were overmodified as judged by the diagonal spots, except for α1(I)CB6. The peptide pattern of the α1′(I)-dimer was identical with that of α1(I) chain except for the absence of the α1(I)CB7 (open arrow) and the presence of a spot corresponding to an α1(I)CB7-dimer (filled arrow), which, when electrophoresed under reducing conditions, runs as a regular α1(I)CB7 peptide (result not shown).

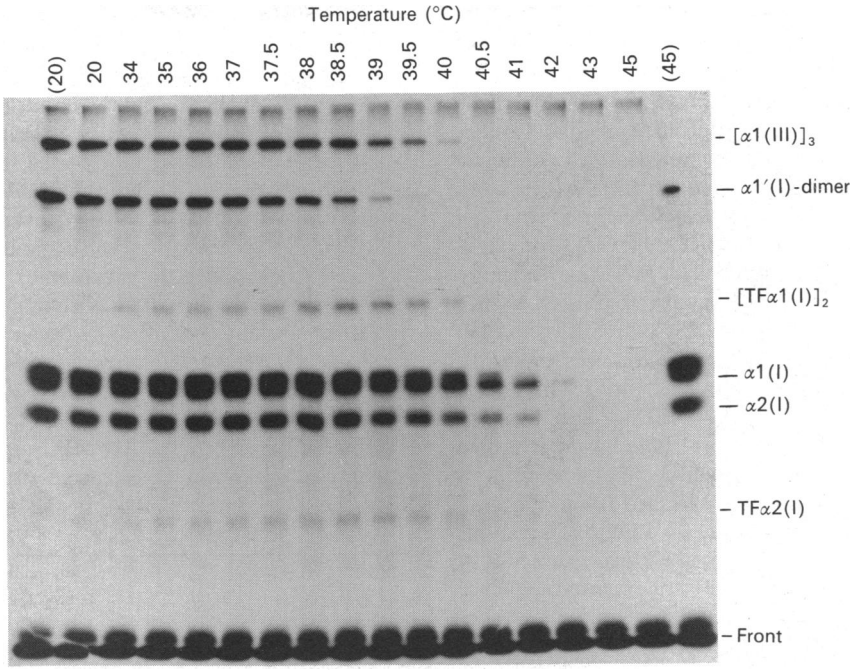

Fig. 2. Thermal stability of the proband's collagen

The 'melting' temperature (T_m) of the abnormal collagen containing two mutant α1(I) chains was decreased to 38.5 °C, as judged by the disappearance of the α1′(I)-dimer, and the T_m of collagen containing only one mutant chain was 40.0–40.5 °C, as judged by the disappearance of a doublet forming overmodified α1(I) chains. In parallel with the disappearance of the α1(I)-dimer, two new components appear, $[TF\alpha'(I)]_2$ and TFα2(I), in a 2:1 ratio with molecular masses of 140 and 70 kDa respectively. These bands apparently correspond to an unfolding intermediate (see the text) that has a T_m similar to that of collagen containing only one mutant chain, i.e. 40.0–40.5 °C. Note that the T_m values of regular collagens I and III are 41 °C and 39.5 °C respectively, and thus are normal. The temperature in parentheses indicate samples that were not exposed to trypsin. TF stands for trypsin-cleavage fragments of the α1(I) and α2(I) chains.

the reducible α1′(I) dimer produced by her cells *in vitro* (results not shown). In addition, there was a 270 kDa reducible component reminiscent of that found in a patient with a cysteine-for-glycine substitution at position 988 (Steinmann *et al.*, 1984; Cohn *et al.*, 1986), the nature of which remains to be determined.

Electron microscopy of the skin

Electron microscopy of the skin indicated that many of the fibroblasts had an enlarged endoplasmic reticulum filled with granular material. Also, there was a decrease in the diameter of the collagen fibrils. In the papillary dermis the mean diameter was 38 ± 6 nm compared with 47 ± 4 nm (range 39–56 nm) in controls. In the reticular dermis the mean diameter was 68 ± 8 nm compared with 88 ± 5 nm (range 77–99 nm) in controls.

Prenatal diagnosis

Procollagen I synthesized by the parents' skin fibroblasts was normal, suggesting that the proband's condition was caused by a new mutation. Because we could not rule out parental mosaicism on the basis of protein and molecular data obtained from the parents' fibroblasts, they were informed that the recurrence risk was 5–7% (Thompson *et al.*, 1987; Byers *et al.*, 1988; Constantinou *et al.*, 1990). Therefore, during the next pregnancy, prenatal diagnosis was performed on a chorion villus biopsy. The cells had a 46 XY karyotype. The collagen pattern, after radiolabelling the villus biopsy, was indistinguishable from that of control chorion villus samples. Specifically, the pathognomonic α1′(I)-dimer was not present and there was no overmodification of the α1(I) and α2(I) chains (A. Superti-Furga & B. Steinmann, unpublished work). Ultrasound monitoring of the fetus was normal, and the mother delivered a healthy boy.

Nucleotide sequence of the mutation found in the patient's mRNA

To detect the mutation in the patient's mRNA, a double-stranded cDNA was prepared and used as template for the PCR. Since the thermal-stability experiments suggested local unfolding of the triple helix around residue 700, primers were designed to span codons for amino acid residues 358–851 of the α1(I) chain and amino acid residues 396–730 of the α2(I) chain. The PCR products were cloned to M13 and sequenced. The results revealed a single-base mutation that changed the codon GGT for glycine-691 of the α1(I) chain to TGT, a codon for cysteine (Fig. 4). The presence of the mutation in the codon for glycine-α1-691 was used to distinguish the two alleles in M13 clones from the overlapping PCR products from primer sets II and III (see the Materials and methods section) of the proα1(I) chain. From primer set II, three of five M13 clones contained the mutation and two contained the normal sequence. From primer set II, eight clones from the allele containing the mutation and 11 clones without the mutation were sequenced. In addition, 25 clones from primer set I were sequenced. The results indicated that there was no second mutation in codons for amino acid residues 358–851 of the proα1(I) chain. To exclude a second mutation in the corresponding region of the gene for proα2(I) chains (*COL1A2*), two separate preparations of PCR products containing codons for amino acid residues 516–730 were cloned and sequenced. Analysis of 19 clones from primer set I and 24 clones from primer set II did not reveal a second mutation.

Confirmation of the mutation by allele-specific hybridization

To confirm the mutation at glycine-α1-691, the proband's

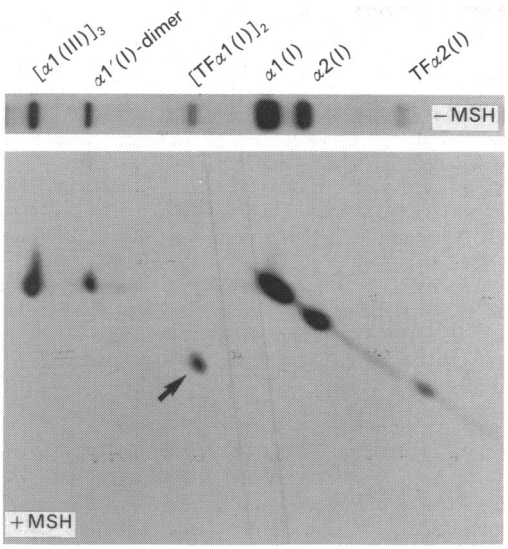

Fig. 3. Characterization of the collagen fragments generated by trypsin at 38.5 °C

On the basis of the information obtained in the 'melting' experiment (Fig. 2), the collagens were heated to 38.5 °C and then treated with trypsin. At this temperature the abnormal collagen containing two mutant α1′(I) chains is partially unfolded and therefore is cleaved into the corresponding trypsin-cleavage fragments $[TF\alpha1'(I)]_2$ and TFα2(I). The ratio of the α1′(I)-dimer to $[TF\alpha1'(I)]_2$ is 1:1 (see horizontal lane; 5% gel, unreduced −MSH). When the gel strip was cut out and the collagens were electrophoresed in the second dimension under reducing conditions (+MSH) (lower panel), the α1′(I)-dimer migrated as regular α1(I) chains, and $[TF\alpha1'(I)]_2$ was reduced and migrated with an apparent molecular mass of 70 kDa (arrow); again TFα1′(I) and TFα2(I) were in a 2:1 ratio. It was therefore concluded that the mutation induces a local unfolding of the helix in a region close to position 700 (e.g. at 704, where there is an arginine residue in both α chains) and that the cysteine substitution must be located *N*-terminal to the cleavage site (see the text).

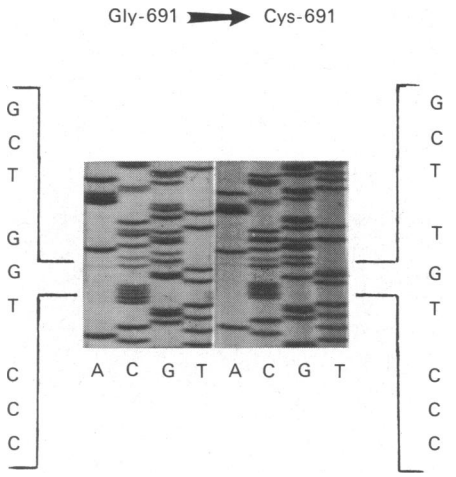

Fig. 4. Nucleotide sequence of the region around glycine-α1-691 and of the single-base mutation cysteine-α1-691

The sequence was obtained by using cDNA as a template for the PCR and cloning the products into bacteriophage M13.

genomic DNA was used as a template for the PCR with a set of primers extending from intron 35 to exon 40. The PCR products were of the expected size of about 0.95 kb. The products were

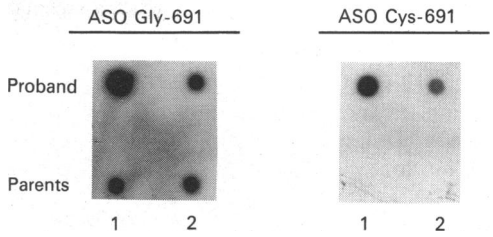

Fig. 5. Allele-specific oligonucleotide hybridization of genomic DNA and cDNA

Genomic DNA and cDNA was used as the template from the proband, and genomic DNA was used as the template from the proband's parents. Top row: genomic DNA (1) and cDNA (2) of the proband's fibroblasts hybridized with the allele-specific oligonucleotide for the normal sequence (ASO Gly-691) and for the mutated sequence (ASO Cys-691). Bottom row: genomic DNA of the proband's father (1) and mother (2) only hybridized with the oligonucleotide specific for the normal sequence (ASO Gly-691).

dot-blotted on to a nitrocellulose filter and used for allele-specific oligonucleotide hybridization. As indicated in Fig. 5, PCR products obtained by using the proband's DNA as template hybridized with both an oligonucleotide containing the normal sequence (ASO Gly-691) and an oligonucleotide containing the mutation for glycine-691 (ASO Cys-691).

In parallel experiments, genomic DNA from the proband's parents were used as template for the PCR and the products were tested by allele-specific hybridization. As indicated in Fig. 5, the PCR products from the proband's parents hybridized only with the oligonucleotide containing the normal sequence (ASO Gly-691).

DISCUSSION

The folding of the triple helix of type I collagen and similar collagens is unusual because the individual α chains do not fold into any defined structure. Instead, the folding first requires association of three proα chains through their globular C-propeptides (see Prockop & Kivirikko, 1984; Prockop, 1990; Engel & Prockop, 1991). The association of the C-propeptides provides correct chain selection and registration of -Gly-Xaa-Yaa- sequences at the *C*-termini of the α-chain domains so that a nucleus of triple-helical structure can form. The nucleus of triple helix is then propagated toward the *N*-terminus of the molecule (Bächinger *et al.*, 1980; Engel, 1987; Prockop, 1990; Engel & Prockop, 1991). The unidirectional folding of the collagen triple helix was directly demonstrated by refolding experiments *in vitro* with type III collagen that contains interchain disulphide bonds at the *C*-terminus of the triple helix (Bächinger *et al.*, 1980). More recently, it was directly demonstrated for the folding of type IV collagen that contains natural interruptions in this repeating -Gly-Xaa-Yaa- sequence of its α chains (Doelz *et al.*, 1988; Doelz & Engel, 1990). Unidirectional folding of the triple helix of type I procollagen has been difficult to demonstrate directly, but unidirectional folding of type I procollagen is consistent with observations that mutations changing the primary structure of the protein usually cause post-translational over-modification of regions that are *N*-terminal but not of regions that are *C*-terminal to the site of the mutation (Byers, 1990; Kuivaniemi *et al.*, 1991).

The mutation examined here is similar to a series of previously reported single-base mutations that converted a glycine codon in the triple-helical domain of type I procollagen to a codon for a bulkier amino acid and caused a lethal variant of osteogenesis imperfecta (see Steinmann *et al.*, 1984; Prockop, 1990; Byers,

1990; Kuivaniemi *et al.*, 1991). One observation, however, was unusual. The substitution of cysteine for glycine-α1-691 made the procollagen abnormally susceptible to cleavage by trypsin to an intermediate fragment of about 700 residues per chain that still contained the cysteine residue, as demonstrated by the presence of a disulphide-linked species of the fragment. Therefore the cysteine-α1-691 altered the conformation of the triple helix *C*-terminal to the mutation so as to expose trypsin-sensitive residues, probably arginine-α1-704 and arginine-α2-704 (Bernard *et al.*, 1983*a*,*b*), that are not susceptible to trypsin in normal type I procollagen and collagen. The results therefore provide one of the first indications that glycine substitutions in type I procollagen can alter the conformation of the triple helix *C*-terminal to the site of the mutation. Similar results have recently been observed with another glycine substitution in the α1(I) chain (Tsuneyoshi *et al.*, 1990; Westerhausen *et al.*, 1990*b*).

After cleavage *C*-terminal to the cysteine-α1-691, the resulting fragment of about 700 residues was more stable to further trypsin digestion than the A fragment of 776 residues from normal type I collagen. Similar intermediates with increased thermal stability were previously observed with other glycine substitutions in proα1(I) chains but were difficult to explain (see Vogel *et al.*, 1987; Westerhausen *et al.*, 1990*a*,*b*). The increased stability of the 700-residue fragment observed here may well be explained by the stabilizing effect of the disulphide bond. Alternatively, cleavage of the molecule at about residue 700 may selectively remove helix-destabilizing triplets from the molecule and thereby make the remaining fragment more stable than the normal collagenase A fragment (see Westerhausen *et al.*, 1990*a*).

This work was supported by grants from the National Institutes of Health (AR38188), the Lucille P. Markey Charitable Trust, the March of Dimes/Birth Defects Foundation, the Swiss National Science Foundation (NF 32-27884.89) and the E.C. Concerted Action on Heritable Connective Tissue Disorders 1990–1992. The skilful technical assistance of Mathis Zopfi is acknowledged.

REFERENCES

Bächinger, H. P., Bruckner, P., Timpl, R., Prockop, D. J. & Engel, J. (1980) Eur. J. Biochem. **106**, 619–632

Bernard, M. P., Myers, J. C., Chu, M.-L., Ramirez, F. & Prockop, D. J. (1983*a*) Biochemistry **22**, 1139–1145

Bernard, M. P., Chu, M.-L., Myers, J. C., Ramirez, F., Eikenberry, E. F. & Prockop, D. J. (1983*b*) Biochemistry **22**, 5213–5223

Bruckner, P. & Prockop, D. J. (1981) Anal. Biochem. **110**, 360–368

Byers, P. H. (1990) Trends Genet. **6**, 293–300

Byers, P. H., Tsipouras, P., Bonadio, J. F., Starman, B. J. & Schwartz, R. C. (1988) Am. J. Hum. Genet. **42**, 237–248

Cohn, D. H., Byers, P. H., Steinmann, B., Gelinas, R. E. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 6045–6047

Constantinou, C. D., Pack, M., Young, S. B. & Prockop, D. J. (1990) Am. J. Hum. Genet. **47**, 670–679

Doelz, R. & Engel, J. (1990) Ann. N.Y. Acad. Sci. **580**, 421–424

Doelz, R., Engel, J. & Kühn, K. (1988) Eur. J. Biochem. **178**, 357–366

Engel, J. (1987) Adv. Meat Res. **4**, 145–158

Engel, J. & Prockop, D. J. (1991) Annu. Rev. Biophys. Biophys. Chem. **20**, 137–152

Gubler, U. & Hoffman, B. J. (1983) Gene **25**, 263–269

Kadler, K. E., Torre-Blanco, A., Adachi, E., Vogel, B. E., Hojima, Y. & Prockop, D. J. (1991) Biochemistry **30**, 5081–5088

Kuivaniemi, H., Sabol, C., Tromp, G., Sippola-Ehiele, M. & Prockop, D. J. (1988) J. Biol. Chem. **263**, 11407–11413

Kuivaniemi, H., Tromp, G. & Prockop, D. J. (1991) FASEB J. **5**, 2052–2060

Laemmli, U. K. (1970) Nature (London) **227**, 680–685

Laskey, R. R. & Mills, A. D. (1975) Eur. J. Biochem. **56**, 335–341

Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor

Piez, K. A. (1984) in Extracellular Matrix (Piez, K. A. & Reddi, A. H., eds.), pp. 1–40, Elsevier, New York

Prockop, D. J. (1990) J. Biol. Chem. **265**, 15349–15352

Prockop, D. J. & Kivirikko, K. I. (1984) N. Engl. J. Med. **311**, 376–386

Prockop, D. J., Vogel, B. E., Doelz, R., Engel, J., Hojima, Y. & Kadler, K. E. (1989) Springer Ser. Biophys. **3**, 292–301

Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A. & Arnmeim, N. (1985) Science **230**, 1350–1354

Sanger, F., Nicklen, S. & Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5463–5467

Steinmann, B., Rao, V. H., Vogel, A., Bruckner, P., Gitzelmann, R. & Byers, P. H. (1984) J. Biol. Chem. **259**, 11129–11138

Steinmann, B., Superti-Furga, A. & Royce, P. M. (1988) Ann. N.Y. Acad. Sci. **543**, 47–61

Studencki, A. B. & Wallace, R. B. (1984) DNA **3**, 7–15

Superti-Furga, A. & Steinmann, B. (1988) Biochem. Biophys. Res. Commun. **150**, 140–147

Thompson, E. M., Young, I. D., Hall, C. M. & Pembrey, M. E. (1987) J. Med. Genet. **24**, 390–405

Tromp, G., Kuivaniemi, H., Stacey, A., Shikata, H., Baldwin, C. T., Jaenisch, R. & Prockop, D. J. (1988) Biochem. J. **153**, 919–922

Tsuneyoshi, T., Constantinou, C. D., Mikkelson, M. & Prockop, D. J. (1990) Am. J. Hum. Genet. **47**, A240

Vogel, B. E., Minor, R. R., Freund, M. & Prockop, D. J. (1987) J. Biol. Chem. **262**, 14737–14744

Vogel, B. E., Doelz, R., Kadler, K. E., Hojima, H., Engel, J. & Prockop, D. J. (1988) J. Biol. Chem. **263**, 19249–19255

Westerhausen, A., Kishi, J. & Prockop, D. J. (1990*a*) J. Biol. Chem. **265**, 13995–14000

Westerhausen, A., Constantinou, C. D. & Prockop, D. J. (1990*b*) Am. J. Hum. Genet. **47**, A242

Williams, C. J. & Prockop, D. J. (1983) J. Biol. Chem. **258**, 7721–7728

Received 18 February 1991/10 May 1991; accepted 23 May 1991