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# A novel Gly to Arg substitution at position 388 of the $\alpha$1 chain of type I collagen in lethal form of osteogenesis imperfecta✪

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**Cultured skin fibroblasts from a proband with a lethal form of osteogenesis imperfecta produce two forms of type I collagen chains, with normal and delayed electrophoretic migration; collagen of the proband's mother was normal. Peptide mapping experiments localized the structural defect in the proband to $\alpha$1(I) CB8 peptide in which residues 123 to 402 are spaned. Direct sequencing of amplified cDNA covering this region revealed a G to A single base change in one allele of the $\alpha$1(I) chain, that converted glycine 388 to arginine. Restriction enzyme digestion of the RT-PCR product was consistent with a heterozygous *COL1A1* mutation. The novel mutation conforms to the linear gradient of clinical severity for the $\alpha$1(I) chain and results in reduced thermal stability by 3°C and intracellular retention of abnormal molecules.**

Osteogenesis imperfecta (OI) is a dominantly inheritable disorder of the connective tissue characterized by brittleness of bone. It is a heterogeneous disease with a wide range of clinical phenotypes, from barely detectable to perinatal lethal. Four OI subtypes are recognized: mild (type I), lethal (type II), severely deforming (type III) and moderately severe (type IV) (Sillence *et al.*, 1979). Almost all forms of OI are caused by defects in type I collagen which is the major structural protein of bone, skin and tendons. The majority of individuals (>90%) with OI have mutations in the *COL1A1* and *COL1A2* genes that encode the pro$\alpha$1 and pro$\alpha$2 chains of type I procollagen, respectively (Kuivaniemi *et al.*, 1991; Prockop, 1992). These mutations include genomic deletions, splicing mutations causing exon skipping or deletions of different length. Most of the cases of lethal OI, however, result from point mutations that

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**Abbreviation:** OI, osteogenesis imperfecta.

produce substitutions for single glycine residues within the Gly-X-Y repeat units characteristic of the collagen triple helix. Replacement of a glycine residue with a bulkier amino acid distorts conformation of the triple helix, because each of the glycine residues is located in the sterically restricted position in the center of the triple helix. Mutations that affect quantitatively production of type I collagen are responsible for the mildest form of disease (Willing *et al.*, 1996). On the other hand, point mutations, splicing mutations, deletions and insertions, which produce qualitatively abnormal collagen molecules, have been found to be the cause of the severe and lethal forms of OI (Cole, 1994).

In our previous studies with fibroblasts from a proband with lethal form of OI we have shown that decreased collagen synthesis and altered cell growth can be caused by reduced activity of prolidase, which may be involved in producing the lethal phenotype (Galicka *et al.*, 2001). Here, we describe the molecular defect responsible for the disease. We have identified a novel Gly388Arg substitution arising from a mutation in the *COL1A1* gene of the proband occuring in a highly mutagenic CpG dinucleotide.

## MATERIALS AND METHODS

***Clinical material.*** Ultrasonographic examination at 22 weeks of gestation revealed deformed short femurs and poorly ossified skull of the fetus. The male proband was delivered and died 3 days later. The clinical and radiologic picture was characteristic of OI type II. The proband showed multiple fractures of long bones and ribs. The skull was not ossified, membraneous and very soft. He was from the first pregnancy of apparently normal nonconsanguinous Polish parents, a 24-year-old mother and 25-year-old father. A second pregnancy resulted in a normal child.

***Biochemical analysis.*** Skin fibroblast cultures were established from the proband, his mother and normal control individual as described by Bonadio *et al.* (1985). The biopsies were obtained with the approval of the Ethics Committee of the Medical Academy (Białystok, Poland) and with written parental consent.

Confluent fibroblast cultures were labeled with 50 $\mu$Ci/ml L-[5-$^3$H]proline (28 Ci/mmol, Amersham Corp.) for 18 h in fresh Dulbecco's modified Eagle medium containing 50 $\mu$g/ml of ascorbic acid. Procollagens from the cell layer and medium were harvested separately (Bonadio *et al.*, 1985) and converted to collagens by limited proteolysis (4 h at 4°C) with pepsin (50 $\mu$g/ml). The collagen chains were separated on 5% SDS/urea/polyacrylamide gel (Bonadio *et al.*, 1985; Laemmli, 1970) which was processed for fluorography with Amplify (Amersham Corp.) as described by Laskey & Mills (1975). To analyze unglycosylated procollagens, fibroblasts were labeled as described above, but for 4 h and in the presence of 0.3 mM $\alpha,\alpha'$-dipyridyl.

Cyanogen bromide treatment of proteins in polyacrylamide gel and separation of peptides in the second dimension by 12.5% SDS/PAGE were performed as described by Bonadio *et al.* (1985).

Thermal stability of collagen molecules was determined as described by Wenstrup et al. (1991). Briefly, the collagen was incubated at a range of temperatures from 20 to 43°C for 10 min in a Perkin-Elmer thermal cycler, rapidly cooled to 20°C and then exposed to a mixture of trypsin and chymotrypsin for 2 min. Digestion products were analyzed electrophoretically as described above. Radioactivity in the protein bands was determined by excision of the individual bands and scintillation counting.

***Molecular analysis.*** Total RNA was isolated from cultured skin fibroblasts using the guanidinium/phenol method (Chomczynski & Sacchi, 1987). About 1 $\mu$g of total RNA was used to synthesize single-stranded cDNA using RT-PCR (RNA PCR kit, Perkin-Elmer). For the proband and control, fragments encoding the C-half of $\alpha$1(I) CB8 (aa 261–404) were amplified by RT-PCR. First-strand syn-

thesis utilized the reverse primer located at nt 1836–1865 of the cDNA sequence and a 15 min incubation with *MuLV* reverse transcriptase at 42°C. The forward primer located at nt 1434–1463 of the cDNA sequence was added for PCR amplification. The conditions of amplification were as follows: one cycle of 1 min at 94°C, 30 s at 68°C, and 1 min at 72°C, followed by 35 cycles of 30 s at 94°C, 30 s at 68°C, and 45 s at 72°C. The reaction product was submitted to agarose gel electrophoresis and column purification (GenElute Agarose spin column, Sigma), and then used for asymmetric amplification with the reverse primer in conditions described above. The PCR product obtained was directly sequenced with T7 Sequenase (Amersham) using the dideoxy chain termination method (Sanger *et al.*, 1977). As sequencing primer a forward primer (Perkin-Elmer), the 30-mer 5′ CCC AAG GGT CCC GCT GGT GAA CGT GGT TCT 3′ was used. As an additional control for the PCR reaction, RNA from control fibroblasts was also amplified and sequenced as described above.

The mutation detected during sequencing of the cDNA was confirmed by digesting RT-PCR products containing the mutation site, from two independent preparations of RNA, with the restriction endonuclease *Ava*II. To generate this product the following primers were used: the forward primer located at nt 1759–1788 and the reverse located at nt 1836–1865 of the cDNA. The digestion products were separated on a 10% polyacrylamide gel.

## RESULTS

### Electrophoretic analysis of collagen

Electrophoretic analysis of type I collagen synthesized by fibroblasts of the proband showed in addition to normal collagen chains the presence of $\alpha$1(I) and $\alpha$2(I) chains with delayed electrophoretic migration (Fig. 1A). The abnormal collagen was detected in both medium and cell layer, but in contrast to the control cells which secreted collagen efficiently, the OI fibroblasts accumulated it intracellularly. These abnormalities appear to be specific for type I collagen, since type III collagen had normal electrophoretic migration and normal secretion (Fig. 1A). This suggests that retention of abnormal collagen is not a general secretion problem of the OI fibroblasts. The collagen molecules of the proband's mother were freely secreted into the medium and migrated as normal. When collagen of the proband was synthesized in the presence of $\alpha$,$\alpha'$-dipyridyl, which blocked lysyl and prolyl hydroxylation, only normally migrating collagen chains were present (not shown). This demonstrates that the delayed electrophoretic mobility resulted from increased post-translational modification of the collagen chains, rather than the increased length of the polypeptide.

To obtain an approximate localization of the mutation along the length of the chain, we determined the gradient of CNBr peptides with delayed electrophoretic migration. When we examined the cyanogen bromide peptides, only the CB8 peptide of the proband $\alpha$1(I) chain migrated with a slanted pattern as compared to the mother and control chains (Fig. 1B). This suggests localization of the mutation in the amino-terminal 40% of the chain.

### Localization of mutation

The region of the mRNA encoding the C-terminal half of the CB8 peptide (aa 261–404) of $\alpha$1(I) collagen was amplified using RT-PCR and asymmetric PCR. Direct sequencing of the asymmetric PCR product using the forward primer, from two independent RNA preparations, was performed in duplicate. The sequence corresponding to the first nucleotide of the codon for glycine 388 contained two bands of equal intensity co-migrating in the G and A reaction lanes (Fig. 2). The GGA codon corresponds to the normal

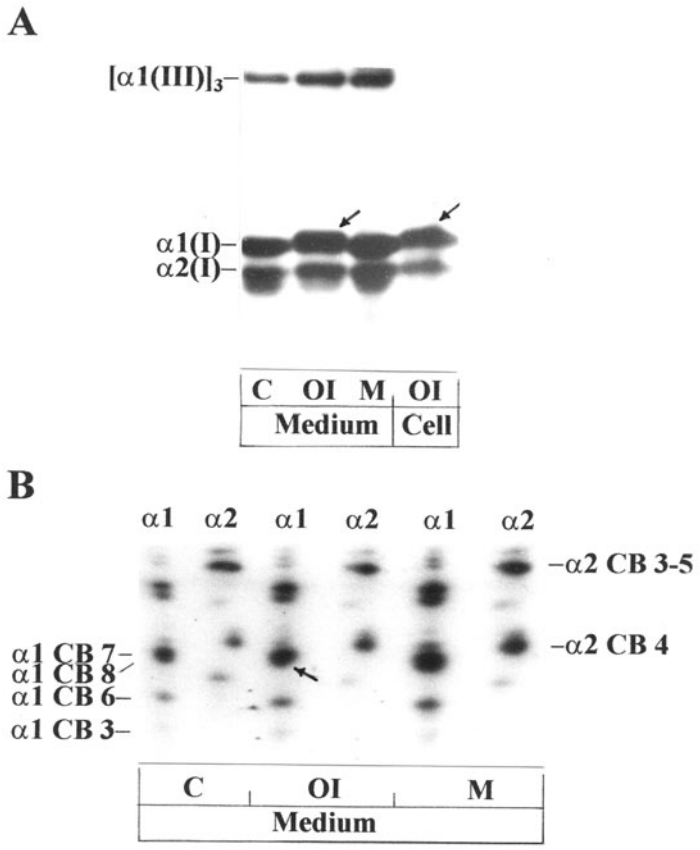


**Figure 1. A. Electrophoretic mobility of collagen chains synthesized by dermal fibroblasts from the proband (OI), his mother (M) and control (C).**

Migration positions of the $\alpha$1(I) and $\alpha$2(I) chains of type I collagen and unreduced $\alpha$1(III) chains of type III collagen are shown. The arrows indicate the abnormal, slowly migrating collagen chains.

**B. Collagen peptides after cyanogen bromide digestion.**

Gel strips were treated with CNBr (50 mg/ml) and the peptides separated by 12.5% SDS/PAGE. The arrow indicates the CB8 peptide of proband's $\alpha$1(I) with a slanted migration pattern.

Gly 388 at that position, while the AGA codon results in an arginine substitution for glycine. The data indicated that the proband was heterozygous for codon 388 of the $\alpha$1(I) procollagen, with one allele coding for glycine and the other for arginine.

The G-to-A-mutation eliminated a restriction site for *Ava*II. To confirm the mutation we amplified the region of cDNA containing the mutation site, from the proband and the normal control. The products were purified and digested with restriction endonuclease *Ava*II. Digestion of the control 108 bp PCR product produced four fragments of 50, 27, 27 and 4 bp, respectively (Fig. 3). When in the proband the mutation was present, a restriction site was abolished, and a novel 77 bp band appeared.

### Thermal stability of OI collagen

Collagen molecules secreted into the culture medium of normal and OI cells were submitted to thermal denaturation followed by trypsin-chymotrypsin digestion (Fig. 4). The melting temperature ($t_m$) of control type I collagen chains was 42°C, whereas the OI collagen chains melted at 39°C, thus showing that the triple helix instability resulted from the presence of abnormal chains in the heterotrimers.

## DISCUSSION

The clinical variability of OI is matched by extensive heterogeneity in the mutations causing the disease. Of the over 200 mutations characterized in unrelated individuals, the majority are substitutions of a charged or bulky amino-acid residue for the obligatory glycines in either the gene for pro$\alpha$1(I) or pro$\alpha$2(I) collagen. Such glycine substitutions have been associated with a dramatic range of clinical severity, from barely detectable to perinatal lethal. However, despite extensive data, a relationship between the location of the substitutions for glycine residues and OI phenotype has proven elusive.

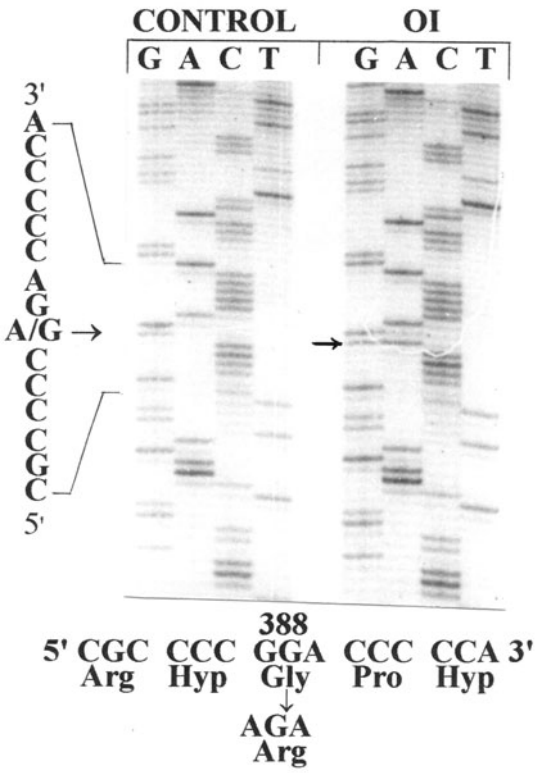


**Figure 2. Direct sequencing of PCR products obtained by asymmetric PCR amplification of $\alpha$1(I) cDNA of the proband (OI) and control.**

The first band of the codon corresponding to glycine 388 is present as a band of equal intensity in both the G and A lanes (indicated by the arrows) which identified the Gly388Arg mutation.

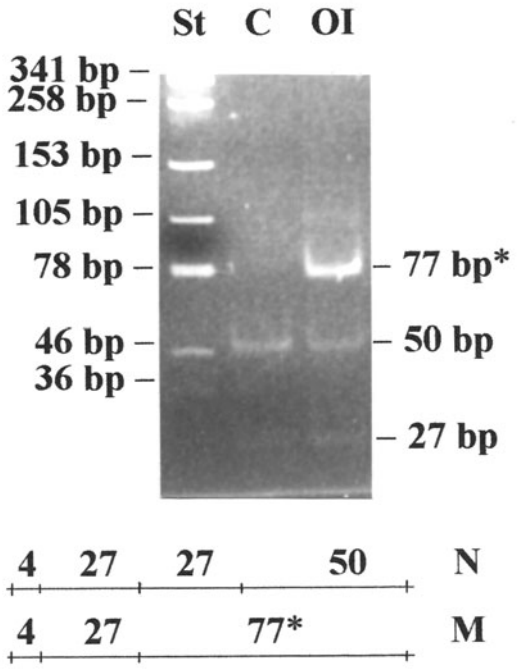


**Figure 3. Restriction digestion with *Ava*II of PCR products amplified from cDNA of the proband (OI) and control (C).**

As predicted by the sequence data, *Ava*II digestion produced fragments of 50, 27, and 4 bp from the normal (N) allele product and fragments of 77, 27 and 4 bp from the mutant (M) allele product (the 4-bp fragment was run off this gel). The presence of cleavage products corresponding to both the normal and mutant allele products in the proband was consistent with a heterozygous G to A mutation at nucleotide 1815 of *COL1A1*. *Dpn*I digest of pUC18 DNA as a molecular size marker (St).

Pruchno *et al.* (1991) postulated that for Gly to Arg substitutions there is a carboxyl- to amino-terminal gradient of phenotypic severity within the $\alpha$1(I) chain. The most $NH_2$ terminal substitutions, G79R (Redford-Badwal *et al.*, 1996; Gat-Yablonski *et al.*, 1997) and G85R (Deak *et al.*, 1991), were found in patients with mild OI type I. The substitutions of arginine for glycine at position 133 resulted in moderately severe type IV (Ward *et al.*, 2001), at position 154 in mild (Zhuang *et al.*, 1996) or severe OI (Pruchno *et al.*, 1991) and at position 172 in severely deforming type III (Mackay *et al.*, 1994). Of the lethal mutations most N terminal mutations occurred at 9211R (Sztrolovics *et al.*, 1994), and all glycine to arginine substitutions located closer to the carboxyl-terminal end were found in lethal cases only. The novel mutation reported here G388R in a type II OI fetus is consistent with this gradient of phenotypic severity.

Both the position within the triple helix, and the nature of the substituting residue determine phenotypic consequences. Bulkier and charged residues, such as arginine, have more destructive consequences than cysteine or serine residues, in replacing glycines within the collagen triple helix. Thus substitution of glycine 211 with cysteine resulted in milder OI type III/IV (Wilcox *et al.*, 1994) whereas Gly211Arg produced lethal OI (Sztrolovics *et al.*, 1994). Similarly, the substitutions of glycine 382 with cysteine (Byers, 1990) or serine (Mackay *et al.*, 1993) resulted in moderately severe type IV, while arginine produced lethal type OI (Cohen-Solal *et al.*, 1996). How-

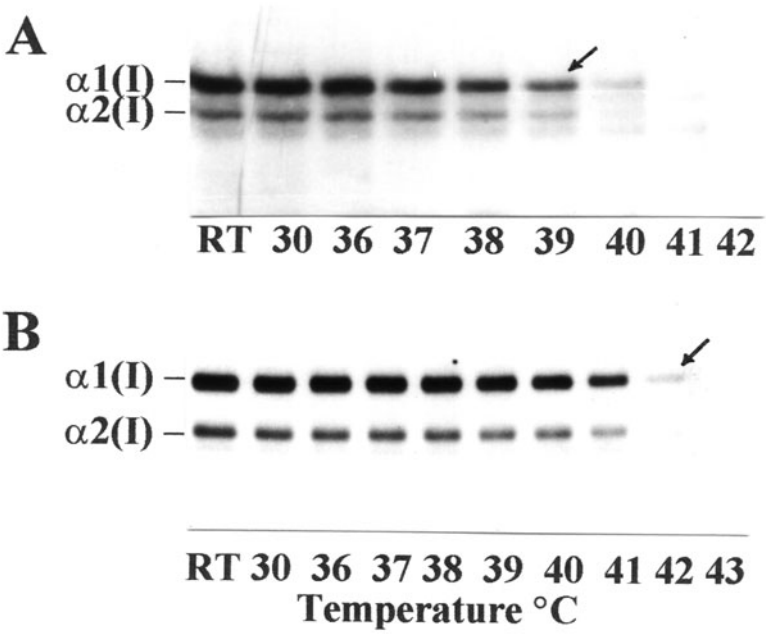


**Figure 4. Thermal denaturation of type I collagen from OI (A) and control (B) fibroblasts.**

The thermal denaturation temperature ($t_m$) was defined as the temperature at which half of the collagen amount underwent degradation.

ever, the phenotypic expression of the disease may be influenced by other yet unknown factors, since an identical substitution of arginine for glycine (G154R) was detected in three unrelated individuals with different types OI (Pruchno *et al.*, 1991; Zhuang *et al.*, 1996). In the reported case we found earlier that the activity of prolidase, which is essential in collagen biosynthesis, and expression of receptors regulating the enzyme activity were decreased, which may be involved in producing the lethal phenotype (Galicka *et al.*, 2001). In general, arginine and asparagine substitutions produced the most drastic consequences on collagen metabolism and stability and hence, the phenotypic outcome. However, whereas all aspartic acid substitutions in pro$\alpha$1(I) seem to be lethal regardless of their location, the arginine substitutions follow a severity gradient as illustrated above.

Baker *et al.* (1989) reported that glycine to arginine substitutions (G391R and G667R) in the $\alpha$1(I) chain were associated with a 1.5°C decrease in stability of collagen. The authors suggested that the relatively small lowering of the melting temperature may reflect the nature of the mutations. However, the novel mutation described here, Gly388Arg located close to the Gly391Arg substitution was associated with a 3°C decrease of helix stability. Furthermore, the substitution of arginine for glycine 85 did not result in fully cooperative melting of type I collagen as in the above cases, but in its unfolding in three discrete segments (Deak *et al.*, 1991). The changed, reduced melting temperature could result from micro-unfolding of a series of independent "cooperative blocks" as postulated by Westerhausen *et al.* (1990). The decreased stability of the proband's collagen molecules by 3°C suggests its increased susceptibility to degradation. In combination with impairment of procollagen synthesis and secretion into media (Galicka *et al.*, 2001) the amount of type I collagen available in the extracellular matrix for fibril formation could be diminished.

As reported for other glycine substitutions, also in this case a G-to-A transition occurred at a CpG dinucleotide, which is a common site of recurrent mutations (Pruchno *et al.*, 1991). CpG dinucleotides are highly mutagenic because of the C-to-T transition caused by methylation and subsequent deamination of 5-methylcytosine. In the *COL1A1* gene, only 26 of 338 glycine codons in the triple helix are susceptible to this mutation mechanism, and of the 13 sites of recurrent mutations, seven occur in CpG dinucleotide sequences (Trummer *et al.*, 2001). For *COL1A1*, apart from cysteine and serine, arginine is predominant

in glycine substitutions in the hot spots. The newly identified glycine to arginine substitution is, so far, an isolated case which occurred at position 388 in a CpG dinucleotide site.

It is likely that the novel mutation reported here is a *de novo* mutation of one pro$\alpha$(I) allele as the parents did not show any clinical evidence of OI nor the mother's protein showed any molecular defects, although parental mosaicism (occurring in up to 5–7% of OI patients) (Zlotogora, 1998) cannot be conclusively ruled out.

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