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Research Article

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A Substitution at a Non-glycine Position in the Triple-Helical Domain of proα2(I) Collagen Chains Present in an Individual with a Variant of the Marfan Syndrome

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Abstract

A substitution for a highly conserved non-glycine residue in the triple-helical domain of the proα2(I) collagen molecule was found in an individual with a variant of the Marfan syndrome. A single base change resulted in substitution of arginine$_{618}$ by glutamine at the Y position of a Gly-X-Y repeat, and is responsible for the decreased migration in SDS-polyacrylamide gels of some proα2(I) chains of type I collagen synthesized by dermal fibroblasts from this individual. Family studies suggest that this substitution was inherited from the individual's father who also produces abnormally migrating proα2(I) collagen chains and shares some of the abnormal skeletal features. This single base change creates a new Bsu36 I (Sau I, Mst II) restriction site detectable in genomic DNA by Southern blot analysis when probed with a COL1A2 fragment. The analysis of 52 control individuals (103 chromosomes) was negative for the new Bsu36 I site, suggesting that the substitution is not a common polymorphism. (*J. Clin. Invest.* 1990. 86:1723–1728.) Key words: mutation • connective tissue • genetic • PCR • fibrillin

Introduction

Type I collagen is the most abundant collagen and the major protein in many tissues including skin, bone, ligament, tendon, sclera, cornea, and blood vessels (reviewed in references 1 and 2). The presence of type I collagen in many different tissues implies that it has diverse functional roles. For example, type I collagen provides tensile strength in bone, skin, and tendon, but normally supports mineralization only in bones and teeth. Certain functions of type I collagen must be related primarily to its structural features, but others, such as support for mineral deposition may depend on its interactions with other matrix macromolecules.

The ubiquity of type I collagen and its functional diversity are reflected in different clinical disorders which are associated with genetic abnormalities that affect type I collagen structure or biosynthesis. The principal manifestation of mutations in type I collagen that alter the sites for extracellular processing of the amino propeptide is severe joint laxity (Ehlers-Danlos; type VII) (3–6). Genetic defects resulting in deficient post-translational lysyl hydroxylation of fibrillar collagen molecules in some tissues produces joint laxity, muscle hypotonia, scoliosis, and may predispose the individual to vascular rupture (7, 8). Osteogenesis imperfecta, a disorder in which the primary clinical effect is osteopenia, is due to mutations that decrease the rate of type I collagen biosynthesis or result in disruption of the triple helix (reviewed in reference 1). Thus analyses of genetic abnormalities of type I collagen or its processing have contributed to defining some of its functional roles.

Although the stringent requirement for glycine at every third position in the triple-helical domain has been made clear by the finding of many glycyl substitutions associated with osteogenesis imperfecta, there is little direct information regarding the function of most non-glycine residues in the triple helical domain. For a few residues, there is evidence of involvement in collagen crosslinking (9) or in degradation (10). But at present, the requirement for most non-glycine residues of the triple-helical domain has only been inferred by relative rates of conservation between species. In this study we report an amino acid substitution at a non-glycyl residue in the triple-helical domain of the proα2(I) collagen molecule. This single base mutation converts the highly conserved arginine$_{618}$ at the Y position of a Gly-X-Y repeat to glutamine, and is responsible for the abnormal migration in SDS-polyacrylamide gels of proα2(I) chains of type I collagen synthesized by cells from a proband with cardiovascular and skeletal features of the Marfan syndrome (11). Family studies show that this substitution was inherited from the proband's father, who shares the same type I collagen phenotype biochemically as well as some of the proband's abnormal skeletal features.

Methods

Clinical summary. Detailed clinical information on the proband has been previously reported (11). In brief, she had progressive lumbar scoliosis noted before age 10. At age 30, aortic dilation and aortic regurgitation was noted. By age 37, aortic dilation resulted in congestive heart failure, and the aortic valve and a portion of the ascending aorta were replaced with a porcine heterograft. Examination at age 39 revealed arachnodactyly and a decreased upper to lower segment ratio. Eye exam showed mild myopia but no lens dislocation.

The patient's father suffered from chronic lung disease and chronic renal failure. He had a history of mild myopia, but no history of joint hyperextensibility or dislocation. Physical exam at age 78 revealed absence of joint hyperextensibility, limb disproportion, and arachno-

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dactyly. He died at age 78 from heart failure thought to be secondary to chronic lung disease. Autopsy revealed an enlarged heart with biventricular hypertrophy; heart valves were thickened, but otherwise normal. The ascending aorta was normal in diameter; there was a moderate amount of atherosclerosis, and aortic atheromas were thought to be calcified to an excessive degree. Postmortem examination of the vertebral column showed there was significant upper thoracic kyphosis with marked irregularities in the vertebral body size and outline.

The proband's mother and sibling are both alive and in relatively good health without evidence of a connective tissue disorder.

Cell strains. Human skin fibroblasts from the proband and family members of the proband were provided by Dr. Peter Byers (University of Washington, Seattle). The cells were grown to confluence in Dulbecco's modified Eagle's medium buffered to pH 7.4, with 24 mM sodium bicarbonate and 25 mM Hepes, and supplemented with 20% calf serum (Gibco Laboratories, Grand Island, NY) that had been heat inactivated for 30 min at 56°C. The atmosphere was maintained at 5% carbon dioxide per 95% air at 37°C.

Preparation and separation of collagens synthesized by cultured cells. The cultured dermal fibroblasts were seeded at a density of 250,000 per 35 mm tissue culture dish 24 h before labeling. Cultures were then washed twice with PBS and preincubated in serum-free medium containing 100 μ/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, 50 μM ascorbic acid. After 3–4 h of preincubation, the medium was replaced with 700 μl of serum-free medium with ascorbate, containing 100 μCi 2,3,4,5-[^{3}H]proline, and incubated for an additional 16 h. Then [^{3}H]proline-labeled procollagens were harvested and α chain-sized molecules were prepared by partial digestion with pepsin. The α chains were separated by SDS-PAGE in a 2 M urea, 5.0% polyacrylamide gel as previously described (12).

Synthesis and cloning of double-stranded cDNA. Total RNA was isolated from skin fibroblasts which had been seeded at a density of 400,000 per 100 mm diam tissue culture dish and grown to confluence. Before isolation, cultures were washed twice with PBS and incubated for 72 h in medium containing 100 μM L-ascorbic acid. Total RNA was isolated using the procedure described by Chomczynski and Sacchi (13).

Poly A RNA was prepared from total RNA by oligo-dT chromatography (14) and used to synthesize double-stranded cDNA (15) with the following modification. For first-strand synthesis, the poly A RNA (9.0 μg) was primed using a specific proα2(I) collagen oligonucleotide (20mer: 5'GACTCCAGGACTACCCACAG'3), 1.05 μg, complimentary to nucleotide positions 2867–2886 of the proα2(I) collagen mRNA (16). Second-strand cDNA synthesis proceeded as described (15).

The region of the cDNA which codes for the α2(I)CB3 peptide and the α2(I)CB5 peptide amino-terminal to the fibroblast collagenase cleavage site was amplified by the polymerase chain reaction (PCR)[1] (17) using 20-base oligonucleotide primers. The 3'oligonucleotide primer (5'GCACCAGCAACACCAGGTAG'3) was complimentary to nucleotides 2785–2804 of the proα2(I) cDNA and the 5'oligonucleotide primer (5'CGAGGACCTAATGGAGATGC'3) corresponded to nucleotides 1429–1448. The denaturating, annealing, and extending conditions for PCR were 1.5 min at 94°C, 2 min at 55°C, and 3 min at 72°C, respectively. The PCR products were examined for different size species by agarose gel electrophoresis, using a 1.5% agarose gel 25 cm in length, which was run at 35 V for 48 h. PCR products were then either digested with the Bsu36 I (New England Biolabs, Beverly, MA), which restricts at the same site as Sau I and Mst II, and the products separated by electrophoresis, or they were ligated into Sma I-digested pUC18 or pUC19 vectors (Bethesda Research Laboratories, Bethesda, MD). The ligation mix was then transformed into the *Escherichia coli* strain, DH5α (Bethesda Research Laboratories).

Five positive clones were isolated, their plasmids purified (18), and the double-stranded cDNAs were sequenced by the dideoxy method using T7 DNA polymerase (Sequenase; United States Biochemical Corp., Gaithersburg, MD).

Southern blot analysis. The 3.0-kb Eco RI genomic fragment, containing exons 32–37 of COL1A2 (16, 19) (Fig. 4 *B*), used as the hybridization probe for Southern blot analyses was isolated from a cosmid (20) containing a 27-kb COL1A2 insert. Genomic DNA was isolated from cultured skin fibroblasts or from EBV transformed lymphoblasts by routine methods and 10 μg of genomic DNA was digested with the restriction enzyme Bsu36 I, the fragments separated by agarose gel electrophoresis, transferred to Gene Screen Plus membrane (DuPont-New England Nuclear, Boston, MA), and hybridized according to the manufacturer's recommended procedures.

Results

Byers et al. (11) had previously reported that cells from an individual with a variant of the Marfan syndrome synthesized and secreted both normally migrating α2(I) collagen chains and a population that had an increased apparent molecular weight as determined by SDS-polyacrylamide gel electrophoresis. The region of the abnormal α2(I) collagen chain responsible for the aberrant migration was localized by two-dimensional peptide mapping to the CNBr-cleaved fragment CB3 or CB5 amino-terminal to the fibroblast collagenase cleavage site (triple-helical residues 357–775). We made cDNAs from cultured dermal fibroblasts that were enriched for proα2(I) sequences coding for this peptide. Three modifications of cDNA cloning procedures were used: enhancement of type I collagen mRNA by preincubation with ascorbate, proα2(I)-specific priming of first-strand synthesis, and amplification of a 1,375-bp proα2(I) cDNA fragment by PCR. Primers used for PCR were placed well outside the region determined by peptide mapping to contain the mutation.

To determine if the apparent increased molecular weight of the abnormal proα2(I) chain was due to an insertion of additional coding sequences, the 1,375-bp PCR products were separated by agarose gel electrophoresis under conditions that clearly distinguish molecules differing by less than 30 bp in size. The PCR products from the Marfan proband's cDNA produced only a single size class (data not shown), suggesting that the abnormality in proα2(I) chains from this individual is not due to an insertion of coding sequence.

Since there was no evidence for an insertion of coding sequences, the PCR-amplified cDNA which codes for residues 357–775 was cloned into pUC18 or pUC19 and sequenced. We isolated and sequenced five clones that had the correct size insert (1,375 bp) and the predicted 5' and 3' ends.

The proband was found to be heterozygous for a single nucleotide change that results in an amino acid substitution of glutamine for the arginine at position 618 in the triple-helical domain of the α2(I) collagen chain (Fig. 1). This single nucleotide change, G to A, was predicted to create a new Bsu36 I (Sau I, Mst II) restriction site (recognition sequence CC/TNAGG) in both the cDNA and genomic DNA from this individual. The amplified PCR product and the cDNA clones from the normal and abnormal alleles were digested with the restriction enzyme Bsu36 I and the DNA fragments size fractionated by agarose gel electrophoresis. The cDNA fragments from the normal allele (Fig. 2; lane *6*) show a 557-bp fragment that is digested into 440- and 117-bp fragments in the abnormal allele (Fig. 2; lane *5*) as a result of the new Bsu36 I site (the 117-bp fragment is not visible). Both the 557- and 440-bp

1. *Abbreviations used in this paper:* PCR, polymerase chain reaction.

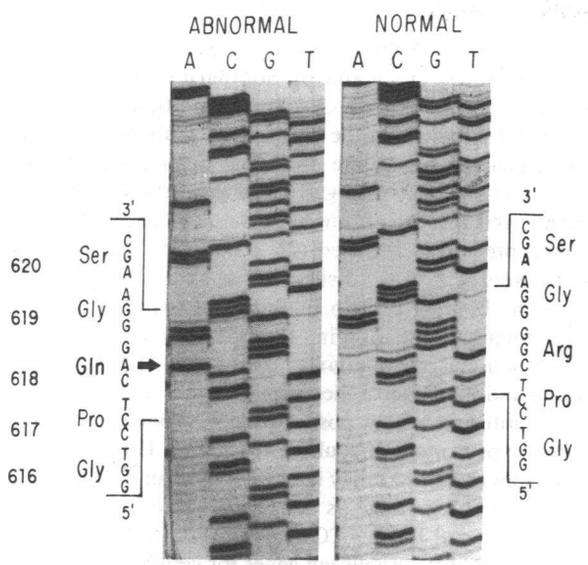

Figure 1. Nucleotide sequence of the proα2(I) collagen cDNA clones from the normal and abnormal alleles. The region of mRNA containing the abnormality had been amplified and cDNA clones in pUC sequenced as described in Methods. The arrow indicates the nucleotide change (*G* to *A*) and the amino acid substitution in the abnormal cDNA compared with the product of the normal allele which corresponds to the published sequence (16).

fragments were present in the Bsu36 I-digested PCR fraction from this proband (Fig. 2).

To determine whether this substitution was an inherited or a de novo mutation, proα2(I) collagen cDNAs were synthesized and amplified from poly A RNA from fibroblast cells of the proband's family. The PCR products were examined for this Bsu36 I site (Fig. 2). The mother and sister of the proband are homozygous for the normal allele, and the father is heterozygous for the normal and abnormal alleles, suggesting that the substitution of a glutamine for an arginine was inherited from the proband's father.

In order to determine if the father also produces an abnormal α2(I) chain, the procollagens synthesized by the proband and the proband's family were examined. [H^3]Proline-labeled collagenous proteins harvested from fibroblasts were treated with pepsin to remove the nontriple-helical domains of the procollagen and then separated by SDS-PAGE (Fig. 3). The proband and the father synthesized a population of α2(I) chains that migrated more slowly than normal, resulting in a broad α2(I) band. This suggests that the arginine to glutamine substitution may account for this abnormal migrating α2(I) chain found in both the father and the proband.

To investigate whether this amino acid substitution was a common polymorphism we examined the proband's family, 52 control caucasian individuals (103 chromosomes), and four individuals (7 chromosomes) with osteogenesis imperfecta who had defined mutations by Southern blot analysis for the novel Bsu36 I restriction site. Only genomic DNA from the proband and the proband's father were positive for the new Bsu36 I site (Fig. 4), which suggests that this mutation is not a common polymorphism. These results also confirmed that both the proband and the proband's father were heterozygous for this amino acid substitution and it was not the result of an artifact in amplification or cloning. In addition, we also examined 17 probands who fulfilled all clinical criteria for the Marfan syndrome (Bsu36 I blots were generously provided by Dr. Clair Francomano, John Hopkins University, Baltimore, MD) for the new Bsu36 I site; it was absent in all of them.

Figure 2. Analysis of Bsu36 I (Sau I)-digested PCR products from proα2(I) collagen cDNA of family members. (*A*) Bsu36 I (Sau I) digests of PCR products amplified from cDNA of the proband (lane *1*) and the proband's mother (lane *2*), father (lane *3*), and sister (lane *4*) were separated by agarose gel electrophoresis. At the top is the family pedigree with an arrow indicating the proband. Sizes (bp) of the Bsu36 I restriction fragments are marked. *The new Bsu36 I restriction site (*B*) and the 440-bp (*A*) Bsu36 I fragment created by the single nucleotide substitution. Lanes *5* and *6* contain Bsu36 I digestion products of the cDNA clones from the proband's normal (lane *6*) and abnormal (lane *5*) alleles. The 387-bp fragment, present in lanes *1–4*, remains attached to the vector DNA in lanes *5* and *6* (not shown). (*B*) The Bsu36 I (Sau I) restriction map of the cloned proα2(I) collagen cDNA fragment from the proband's normal and abnormal allele. Fragment sizes are marked in base pairs below the lines.

Discussion

In this report we demonstrate that the presence of an abnormal proα2(I) chain of type I collagen previously reported in an individual with several features of the Marfan syndrome is due to a substitution at a non-glycine position in the triple-helical domain. Sequence analysis of cDNA's amplified by the polymerase chain reaction and of genomic DNA indicate that a Y-positioned arginine at triple-helical residue 618 is replaced

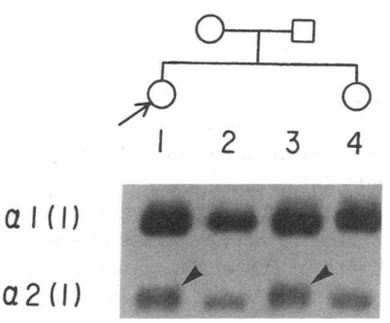

Figure 3. Autoradiofluorograms of [^{3}H] proline-labeled collagenous proteins separated by urea/SDS-PAGE. Procollagens synthesized by dermal fibroblasts from the proband (lane *1*) and the proband's mother (lane *2*), father (lane *3*), and sister (lane *4*) were partially digested with pepsin and separated as described under Methods. At the top is the family pedigree with an arrow indicating the proband. The α1(I) and α2(I) chains are marked. The arrowheads point to the slow migrating population of α2(I) chains.

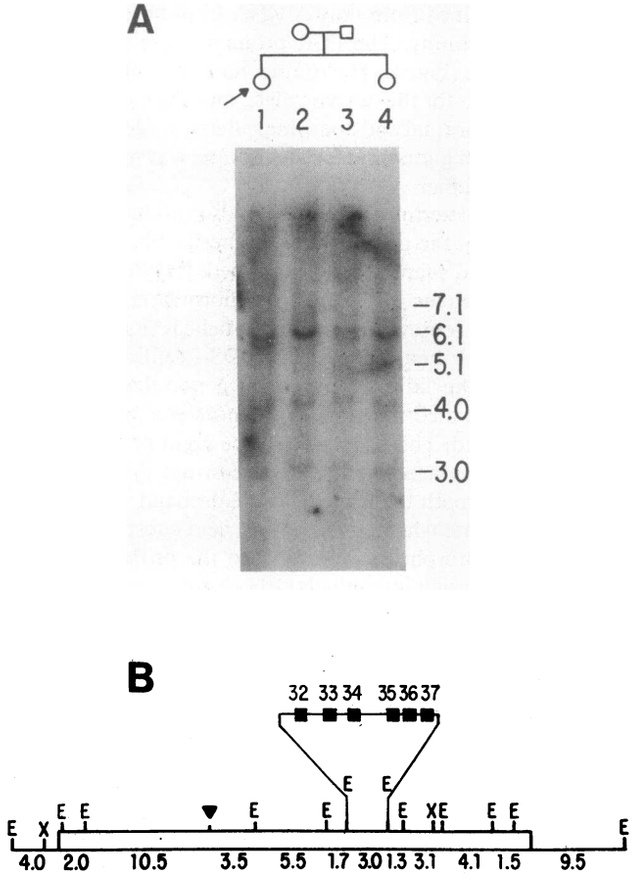

Figure 4. Southern blot hybridizations of the proband and family members' genomic DNA. (*A*) DNA from the proband (lane *1*), the proband's mother (lane *2*), father (lane *3*), and sister (lane *4*) were cleaved with Bsu36 I and probed with a 3.0-kb Eco RI fragment of COL2A1 (*B*). At the top is the family pedigree with an arrow indicating the proband. Sizes are indicated in kilobases. (*B*) An Eco RI restriction map of human COL2A1, the gene is depicted by the open box. *E* and *X* indicate Eco RI and Xho I sites, respectively, with sizes of the Eco RI fragments indicated below the line. (▼) A polymorphic Eco RI site. The 3.0-kb Eco RI fragment used for the hybridization probe contains exons 32–37. The new Bsu36 I site is in exon 35 of COL1A2.

by a glutamine in one COL1A2 allele of this individual. This single base substitution also created a new Bsu36 I restriction site in the genomic DNA, which was used to determine that the individual's father was heterozygous for the substitution. Biochemical and clinical analyses determined that the father shared the type I collagen phenotype, and autopsy studies on the father showed vertebral abnormalities similar to but milder than those of the proband.

There is little doubt that the glutamine for arginine$_{618}$ substitution results in the change in electrophoretic mobility in SDS-polyacrylamide gels that was reported in earlier studies by Byers et al. (11). We isolated cDNAs that contained continuous coding sequences for the entire region of the proα2(I) chain responsible for the altered migration. Two populations of cDNA clones were sequenced in their entirety. They differed only at nucleotide position 2258, which is within the codon for position 618 of the triple helix. Evidence that these two cDNA populations reflected both alleles was confirmed both by sequence analysis of the PCR products of the patient's COL1A2 genomic DNA and by Southern blot analysis of Bsu36 I-digested genomic DNA. The substitution of a neutral glutamine for a basic arginine is consistent with the finding of an acidic charge shift in the abnormal peptide reported by Byers et al. (11). Delayed migration of peptides in SDS-polyacrylamide gels resulting from single amino acid substitutions have been previously reported (21, 22). Most relevant is the report by Noel et al. that a cysteine for arginine substitution in the histidine binding protein, hisJ5625 of *Salmonella typhimurium,* retarded migration in SDS-PAGE (22).

The functional role of arginine$_{618}$ of proα2(I) chain in type I collagen and the possible mechanism by which a nonconservative substitution at that position might produce a Marfan-like phenotype is more difficult to determine. There is genetic evidence that arginine$_{618}$ may have an important functional or structural role. Arginine$_{618}$ is conserved not only across species, in chick, and bovine COL1A2 (23), but also in all the known human fibrillar collagen genes for which sequences are available: COL1A1 (24), COL2A1 (25), COL3A1 (26), and COL5A2 (27), all of which diverged from COL1A2 before diversion of human and chick ancestors. Arginine$_{618}$ also appears to be part of one of many charged domains in the type I collagen molecule that are highly conserved (28). Segment long spacing crystallite studies (2) and later sequence analyses confirm the highly conserved nature of the charged residues in the fibrillar collagens. The substitution of glutamine for arginine$_{618}$ may affect intermolecular alignment during fibril formation, which may explain the finding of increased extractability of the collagen from this individual's skin (11). The finding that arginine$_{618}$ is highly conserved is consistent with data suggesting that this substitution is not a common polymorphism.

Abnormalities in type I collagen have been previously implicated in the Marfan syndrome by reports of accelerated collagen synthesis and turnover (29), abnormal crosslinking (30), and increased solubility of both skin collagen and newly synthesized collagen of patients with Marfan syndrome (31). But linkage studies have excluded COL1A1, COL1A2, COL2A1, and COL3A1 as candidate genes in several families (32–35). Our observation that this substitution is absent in 17 Marfan probands supports the exclusion of COL1A2 by linkage analysis for most Marfan families.

More recently, Godfrey et al. (36) have reported abnormalities in a series of Marfan patients detected by immunofluorescence staining of skin and cultured dermal fibroblasts with antibodies to fibrillin, a component of microfibrils. 24 of 25 individuals with the classical features of Marfan syndrome had abnormal immunofluorescence to anti-fibrillin. The remaining individual with the Marfan clinical phenotype, but with normal elastin-associated microfibrils, was found to have a population of slow-migrating α2(I) chains of type I collagen, similar to those synthesized by cells from the affected individual in this family and her father. Shapiro et al. also reported biochemical abnormalities of α2(I) chains similar to that reported here and apparently similar to that seen by Godfrey et al., in two affected members of a family with scoliosis and osteoporosis (37).

Although the skeletal and cardiovascular abnormalities present in the proband in this report are important characteristics of the Marfan syndrome, they are not restricted to it.

Normal development and function of these tissues are under the control of many genes, and it is possible that subtle alterations in type I collagen structure may play a role in connective tissue disorders that are characterized by familial clustering of cardiovascular and/or musculoskeletal abnormalities (38). Significant intrafamilial differences in clinical severity such as that seen between the proband in this report and her father are seen in many inherited connective tissue disorders, reflecting the complex polygenic nature of extracellular matrix biosynthesis. In addition, the greatly increased incidence in females over males in such connective tissue disorders as mitral valve prolapse and scoliosis is consistent with both the complexity of extracellular matrix biosynthesis and with the finding of limited clinical abnormalities in the father of the female proband in this report.

Finally, these data also suggest that heterozygous substitutions at non-glycine residues in the triple helical domain of type I collagen chains are insufficiently disruptive to bone matrix biosynthesis to produce the osteogenesis imperfecta clinical phenotype (reviewed in reference 1).

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