The substitution of glycine 661 by arginine in type III collagen produces mutant molecules with different thermal stabilities and causes Ehlers-Danlos syndrome type IV

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Abstract

Previous studies have shown that Ehlers-Danlos syndrome type IV (EDS IV) is caused by mutations of type III collagen (COL3A1). Here we have characterised the most amino-terminal glycine substitution so far described in a patient with EDS IV. A combination of peptide mapping and chemical cleavage analysis of cDNA localised the mutation in cyanogen bromide peptide CB5. Sequence analysis showed a G to A mutation, converting glycine 661 to arginine, which was a new dominant mutation. Analysis of type III collagen secreted by cultured fibroblasts showed an overmodified mutant protein with normal thermal stability. However, the intracellularly retained form melted 2°C lower than normal. This indicated that molecules resulting from the same mutation can differ in their thermal stabilities.

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Ehlers-Danlos syndrome type IV (EDS IV) is the most severe of a heterogeneous group of disorders and is usually inherited in an autosomal dominant manner. Affected patients lack or have reduced amounts of type III collagen in their skin. Their cultured skin fibroblasts also secrete a reduced amount of this molecule, mostly retaining the mutant form intracellularly. A number of mutations in the gene (COL3A1) encoding this protein have been found, which include the substitution of glycine residues.

The presence of a glycine at every third amino acid is a prerequisite for the formation of a stable collagen helix. Thus the substitution of a glycine in one of these positions can destabilise the helix and delay its formation, causing excessive post-translational modification N-terminal to the point of the substitution. Analysis of such overmodification has proved useful in mapping the site of single amino acid mutations in collagen molecules.

In type III collagen these amino acid substitutions have produced various effects on the stability of the molecule as judged by thermal induction of helix to coil transition. These effects range from a 2 to 4°C reduction in thermal stability to a normal melting temperature. Here we provide evidence that a single amino acid substitution can produce mutant molecules which differ in their thermal stabilities.

Materials and methods

The full clinical details of the patient have been reported previously by Pope et al (patient 1). She is a non-acrogeric case of EDS IV and is the only affected member of her family.

Dermal fibroblasts were cultured, metabolically labelled, and procollagens and collagens analysed as previously described. Cyanogen bromide peptide mapping of type III collagen was also as previously described.

Total cytoplasmic RNA was isolated from cultured dermal fibroblasts by lysing the cells with NP40 in the presence of vanadyl ribonucleoside complex. First strand synthesis was performed as previously described using the antisense primers described below.

The cDNA encoding CB9 or CB5 was amplified using the oligonucleotide primers CB93, CB95, CB53, and CB55 under conditions previously described. Alternatively the primer E37 was used in conjunction with CB53 to synthesise cDNA encoding the C-terminal half of CB5.

Chemical cleavage analysis was performed as previously described to detect mutations in cDNA encoding CB9 and CB5.

Analysis of DNA obtained from the proband’s blood leucocytes was performed using the restriction enzyme Eco47III. After amplification with the oligonucleotide primers E36:5′GGTGAGAGAGGTGAAACTGGC3′ and AVECO47:5′GGGTCTCTCACCCTCGCAGAGGGAAGTCCCGCTTTTCTC3′, the cDNA was incubated with the enzyme at 37°C for 16 hours and analysed by agarose gel electrophoresis.

The thermal stability of type III collagen synthesised by the proband’s cultured fibroblasts was as previously described.

Results

Initial protein analysis of the labelled procollagens and collagens synthesised by the proband’s cultured fibroblasts was performed by SDS-PAGE. The proα1(III) collagen was poorly secreted (data not shown). After treatment with pepsin, the type III collagen was analysed by electrophoresis with delayed reduction (fig 1A). An extra protein was visible in the proband’s sample, migrating slightly above the normal α1(III) collagen chains. A similar sized protein was also retained intracel-
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Figure 1. Protein analysis. SDS-PAGE analysis of (A) radiolabelled collagens secreted into the medium (M) or retained within the cell layer (C) isolated from a normal control (lanes 1 and 2) and the proband (lanes 3 and 4). Normal (α1(III)M) and slow (α1(III)M) migrating type III collagens are shown, as are the two type I collagen α chains (α1(1) and α2(1)). (B) Type III collagen cyanogen bromide peptides (α1(III) CB5, CB9, CB4-S) from the proband (lane 1) and a normal control (lane 2). The slow migrating CB5 component is indicated as CB5M. Cyanogen bromide peptides of the α1(I) collagen (lane 3) was used as a standard. (C) The arrangement of CB peptides of type III collagen. The position of the oligonucleotides CB93, CB95, CB55, and CB55 are indicated. PstI restriction sites are indicated by P.

Figure 2. cDNA sequences. Antisense sequences from a normal and mutant clone are shown. The sense sequence is written adjacent to the sequences obtained from the gel. The mutated base is indicated by the arrows.

lularly. This was consistent with an overmodified form of the protein caused by a mutation being present. These two species of type III collagen were excised from the gel, cleaved in situ with cyanogen bromide, and then subjected to re-electrophoresis in a second SDS-polyacrylamide gel (fig 1B). Analysis of the pattern showed a spot in the normal position of CB5 and CB9 and one spot migrating slightly slower than the normal products. This contrasted with a previously characterised mutation in CB9, where two abnormally migrating spots were seen. Thus, here, only CB5 appeared to be overmodified while CB9 was normal. Since overmodification normally occurs N-terminal to the site of a mutation, this suggested that a glycine substitution had occurred in CB5 (fig 1C). This was confirmed by chemical cleavage mapping of cDNAs encoding either CB5 or CB9. Whereas a 339 bp cleavage product was detected using the CB5 cDNA, none was detected using the cDNA for CB9 (data not shown). This analysis estimated the site of the mutation to be close to base 2591 of the complete cDNA sequence of type III collagen as described in the EMBL DNA sequence database, accession number X14420.14 A smaller cDNA encoding this region was synthesised, cloned, and sequenced. Multiple clones had the substitution of G by A at base 2584, converting the codon for glycine 661 to one coding for arginine (fig 2). This was confirmed by analysis of genomic DNA. Although the mutation did not destroy a restriction enzyme site, mutant DNA could be distinguished from wild type with the primer AVECO47, which contained a G instead of a C in its sequence (fig 3). After amplification this created an Eco47III restriction enzyme site when the G to A, Gly 661 to Arg mutation was present. Analysis of the proband’s DNA and that of her family confirmed that the mutation only occurred in the proband.

Testing of the type III collagen isolated from the medium showed that the secreted, overmodified form of the protein had the same thermal stability as the normal product, as both proteins were still present at 39°C at approximately equal intensities (fig 4A). However, the overmodified mutant intracellular protein had a reduced thermal stability, being trypsin susceptible after incubation between 37°C and 38°C (fig 4B). In both cases type I collagen acted as an internal control and melted normally.

Discussion

The proband described here has the most N-terminal glycine substitution so far described in a patient with Ehlers-Danlos syndrome type IV. Only the Gly 61915 and 13616 to Arg substitutions in patients with aortic aneurysms are more N-terminal. The Arg 619 substitution did not affect the thermal stability of the secreted type III collagen, but a reduced thermal stability was detected when the collagenase A fragment was analysed. The secreted form of the mutant Arg 661 substitution described here also has normal thermal stability.
Interestingly, whereas the Arg 619 mutation did not affect secretion of the mutant molecule, the Arg 661 substitution caused significant intracellular retention. However, we have shown that the protein retained within the cell melted some 2°C lower than normal and therefore the retained molecules were those which had an altered thermal stability or were pepsin sensitive. Thus for both the Arg 619 and 661 substitutions molecules with normal thermal stability were normally secreted. This contrasts with other COL3A1 mutations. The Gly 910 to Val mutation is partially secreted but has a reduced thermal stability, while a Gly 1018 to Asp mutation has normal stability but is nearly completely retained within the cell. Thus, a correlation between thermal stability of these pepsin resistant triple helices and secretion does not appear to exist.

The difference, seen here, between the secreted and retained form of the mutant protein indicates that molecules resulting from the same mutation can differ in their thermal stabilities. This is perhaps not surprising since type III collagen is a trimeric molecule and mutant forms consisting of one, two, or three abnormal chains will exist, as will the normal product. Presumably this variable composition leads to the differences seen in thermal stability. An alternative explanation for our observations is that some normal homotrimeric of type III collagen from these cells are overmodified and secreted into the medium. However, we think this is unlikely because of the asymmetrical distribution of overmodification, which is seen only in and N-terminal of CB5 and does not affect CB9. This is consistent with the arginine substitution being present in these molecules. As yet we have no data to suggest which particular trimer composition is the more stable.

In summary the proband described here has a new dominant mutation which converts glycine 661 in type III collagen to arginine. This results in two forms of mutant protein. One is secreted and has normal thermal stability, whereas the other is retained intracellularly and melts abnormally between 37°C and 38°C.

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