© 1998 Oxford University Press Human Molecular Genetics, 1998, Vol. 7, No. 6 981–989

Reduced collagen VI causes Bethlem myopathy: a heterozygous *COL6A1* nonsense mutation results in mRNA decay and functional haploinsufficiency

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Received December 22, 1997; Revised and Accepted March 11, 1998

We have identified a new pathogenic mechanism for an inherited muscular dystrophy in which functional haploinsufficiency of the extracellular matrix protein collagen VI causes Bethlem myopathy. The heterozygous *COL6A1* mutation results in a single base deletion from the mRNA and a premature stop codon. The mutant mRNA is unstable, subject to nonsense-mediated mRNA decay, and is almost completely absent both from patient fibroblasts and skeletal muscle, resulting in haploinsufficiency of the α1(VI) subunit and reduced production of structurally normal collagen VI. This is the first example of a muscular dystrophy caused by haploinsufficiency of a structural protein or member of the dystrophin–glycoprotein complex, and identifies collagen VI as a critical contributor to cell–matrix adhesion in skeletal muscle.

INTRODUCTION

The inherited muscular dystrophies are a heterogeneous group of diseases which principally affect skeletal muscle and are characterized by progressive muscle weakness and wasting. Over recent years, as the molecular basis of many of these disorders has been determined, it has become apparent that a common pathogenic mechanism is loss of linkage between the cytoskeleton and the extracellular matrix in skeletal muscle (1). A major contributor to this attachment, which anchors muscle cells in the surrounding basement membrane and is thought to protect the cell membrane from stresses caused by muscle contraction, is the dystrophin–glycoprotein complex (2–4). Mutations in components of this complex are responsible for a variety of muscular dystrophies. The most common muscular dystrophies, Duchenne and Becker, are due to mutations in the cytoskeletal protein dystrophin (5). Associated with dystrophin is the sarcoglycan complex, a group of at least four transmembrane proteins, and mutations in α-, β-, γ- and δ-sarcoglycan result in limb girdle muscular dystrophy 2D, 2E, 2C and 2F respectively (6–9). An additional transmembrane protein, β-dystroglycan, is closely associated with the sarcoglycan complex and its intracellular domain interacts with dystrophin (10). The extracellular protein α-dystroglycan then links the sarcoglycan–β-dystroglycan complex to the surrounding matrix by binding to both β-dystroglycan and the G domain of the basement membrane component laminin (2,11–13). The importance of the basement membrane for normal muscle function is highlighted by mutations in the laminin α2 chain gene, *LAMA2*, which are responsible for laminin-2-deficient congenital muscular dystrophy (14). Thus, mutations in components of both the dystrophin–glycoprotein complex and the basement membrane disrupt the complex, compromise cell–matrix adhesion and result in the dystrophic process.

The range of extracellular matrix molecules important for skeletal muscle stability and muscle cell–matrix adhesion was extended recently with the identification of collagen VI mutations in Bethlem myopathy (15), a mild dominantly inherited disorder with generalized muscle weakness and wasting and contractures of multiple joints (16). Collagen VI is found in the extracellular matrix of virtually all connective tissues where it forms a microfibrillar network. It is abundant in skin and cornea, and is also found in bone and cartilage, and in close association with the basement membrane around nerves and muscle cells (17–19). Three genetically distinct polypeptide chains, α1(VI), α2(VI) and α3(VI), associate intracellularly to form triple-helical collagen VI molecules, the precursors of the characteristic extracellular microfibrils (17). The three reported collagen VI mutations are glycine substitutions within the triple-helical domain of the α1(VI) and α2(VI) chains (15). The mutations disrupt the Gly-X-Y repeat motif which defines the triple helix and, although no collagen VI protein biosynthetic studies or structural analyses were performed, the mutations were assumed to act in a dominant-negative fashion by disturbing collagen VI assembly and resulting in the presence of structurally abnormal microfibrils in the extracellular matrix.

In this study, we have characterized a novel collagen VI mutation in Bethlem myopathy and provide direct biochemical evidence for a new muscular dystrophy disease mechanism, protein haploinsufficiency. We show that a point mutation in

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COL6A1, the gene coding for the α1(VI) subunit, redefines the 5′ boundary of exon 12, resulting in the deletion of a single base from the α1(VI) mRNA and the introduction of a downstream in-frame premature stop codon. The mutant mRNA is unstable, subject to nonsense-mediated mRNA decay, and is almost completely absent from patient fibroblasts. The resultant reduced availability of α1(VI) chains limits the total production and secretion of collagen VI. Thus the phenotype in this family results from reduced synthesis of structurally normal collagen VI. The dominant nature of this mutation identifies collagen VI as a critical component of the skeletal muscle extracellular matrix, and indicates that the presence of appropriate levels of collagen VI in the matrix around muscle cells is essential for normal myotube stability and function.

RESULTS

Bethlem myopathy linked to 21q22.3

We have studied a large Australian family with Bethlem myopathy. The disease was dominantly inherited with onset in early childhood, presenting as muscle weakness with a predominantly proximal distribution and variable, usually slow progression thereafter. Muscle weakness was variable, and joint contractures were seen in only three of the 11 affected individuals. The defect in this family showed complete co-segregation with the variable number tandem repeat markers *D21S156* and *D21S171* at 21q22.3 with two-point lod scores (at θ_{max} = 0.0) of 4.2 and 3.1 respectively. A three-point analysis gave a lod score (at θ_{max} = 0.0) of 5.4. These linkage data established the collagen VI genes *COL6A1* and *COL6A2* as candidate disease genes in this family.

Bethlem myopathy fibroblasts synthesize structurally normal collagen VI

In an attempt to define a collagen VI defect, the entire coding regions of the *COL6A1* and *COL6A2* fibroblast mRNAs were amplified by RT-PCR and directly sequenced. This analysis failed to detect a mutation. Structural disease-causing mutations in other members of the collagen protein family commonly disturb protein folding and oligomeric assembly, and result in increased levels of post-translational proline hydroxylation and glycosylation which can be detected by altered gel migration, increased intracellular degradation and reduced secretion (20). Since collagen VI is abundantly expressed in skin fibroblasts (21) which are readily accessible, control and patient fibroblast cultures were labelled overnight with [^{35}S]methionine and the collagen VI in the cell and medium fractions immunoprecipitated and analysed by SDS–PAGE under reducing conditions. Individual α1(VI), α2(VI) and α3(VI) chains synthesized by the Bethlem myopathy cells migrated normally and were secreted efficiently (Fig. 1a). The normal pathway of collagen VI assembly involves the intracellular assembly of triple-helical monomers containing all three chains to form disulphide-bonded dimers (six chains), and then tetramers (12 chains) which are secreted and associate end-to-end to form microfibrils in the extracellular matrix (17). Further analysis of the collagen VI on non-reducing composite acrylamide–agarose gels demonstrated that, as in control cells, collagen VI tetramers were the major secreted form in the Bethlem myopathy cultures, and these tetramers were also able to form higher order assemblies (Fig. 1b). Thus, both direct sequencing of *COL6A1* and *COL6A2*

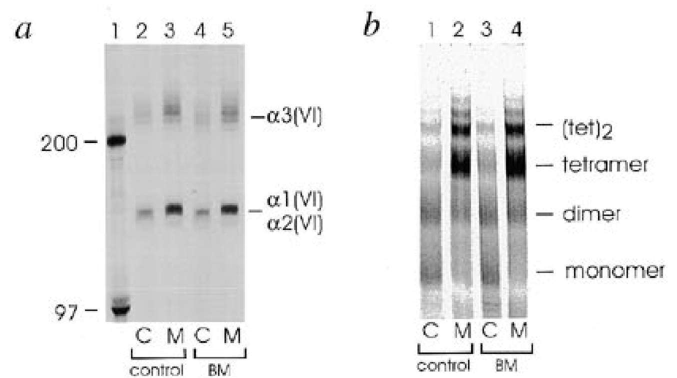

Figure 1. Electrophoretic analysis of collagen VI. Control and Bethlem myopathy (BM) fibroblasts were biosynthetically labelled overnight with [^{35}S]methionine and the collagen VI in the cell (C) and medium (M) fractions immunoprecipitated and analysed under reducing conditions on a 5% polyacrylamide gel (**a**) or without reduction on a compostite 0.5% agarose–2.5% acrylamide gel (**b**). The migration positions of the individual collagen VI subunits α1(VI), α2(VI) and α3(VI) are indicated on the right of (a), and the 200 and 97 kDa molecular weight standards on the left. The collagen VI disulphide-bonded triple-helical monomers, dimers, tetramers and higher order structures [$(tet)_2$] are labelled in (b).

RT-PCR products and electrophoretic analysis of the collagen VI protein synthesized by the Bethlem myopathy cells demonstrated the presence of only normal collagen VI mRNA and protein and failed to provide any indication of a collagen VI structural abnormality.

α1(VI) mRNA is specifically reduced

To determine if patient cells synthesized collagen VI mRNAs of the correct size and in the correct proportions, fibroblast RNA northern blots were probed simultaneously with ^{32}P-labelled α1(VI), α2(VI) and α3(VI) cDNAs. While no abnormally migrating mRNA species were observed, Bethlem myopathy cells showed a specific, ~50% reduction in the level of α1(VI) mRNA relative to α2(VI) and α3(VI) mRNAs (Fig. 2).

A *COL6A1* translation termination mutation

Since nonsense-mediated mRNA decay results in decreased steady-state mRNA levels (22) and is a common but often overlooked consequence of mutations which introduce an in-frame premature stop codon (23), we searched for a *COL6A1* translation termination mutation using the protein truncation test (24). Protein synthesis inhibitors have been shown to reverse the decay of mRNAs containing premature stop codons (25), and so the Bethlem myopathy fibroblasts were treated with cycloheximide prior to RNA extraction in an attempt to stabilize any mRNAs which may have contained a mutation of this class. *COL6A1* sequences coding for the N-terminal domain, the triple helix and part of the C-terminal domain were amplified by RT-PCR using a 5′ primer which included the T7 promoter sequence, a ribosome-binding site and an in-frame translation initiation ATG codon, in addition to the specific priming sequence. The RT-PCR products were transcribed and translated *in vitro* and the resultant radiolabelled proteins analysed on acrylamide gels. The Bethlem myopathy samples contained, in addition to the 82 kDa full-length product, a smaller 37 kDa

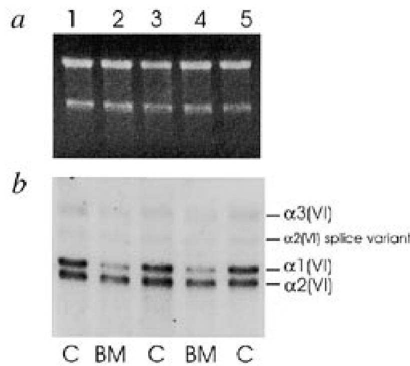

Figure 2. Northern blot of collagen VI mRNAs. Total RNA (5 μg/lane) isolated from three separate control fibroblast lines (C) and the Bethlem myopathy fibroblasts (BM) was separated on a denaturing agarose gel (**a**), transferred to nitrocellulose and hybridized simultaneously to ^{32}P-labelled α1(VI), α2(VI) and α3(VI) cDNAs (**b**). The migration positions of the three mRNA species as well as a relatively rare, larger α2(VI) mRNA which results from alternative splicing are indicated. The Bethlem myopathy cells show a 50% reduction in the level of α1(VI) mRNA relative to α2(VI) and α3(VI).

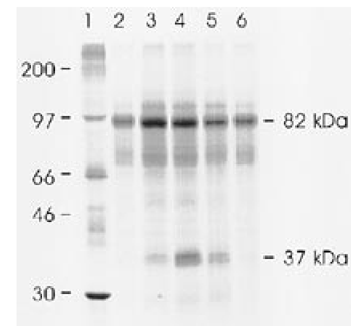

Figure 3. Detection of a *COL6A1* premature termination mutation by the protein truncation test. Bases 34–2290 of *COL6A1* coding sequences were amplified by RT-PCR using a 5′ primer which included the T7 promoter, a ribosome-binding site and an ATG initiation codon. PCR products were transcribed and translated *in vitro* in the presence of [^{35}S]methionine and the resultant protein products analysed on a 14% polyacrylamide gel. Radiolabelled proteins were detected by fluorography. The migration positions of molecular weight standards (lane 1) are indicated on the left, and the 82 kDa full-length translation product and truncated 37 kDa product on the right. Proteins resulting from translation of control RT-PCR products are shown in lanes 2 and 6. Bethlem myopathy fibroblasts were treated with 100 μg/ml cycloheximide for 0 or 8 h (lanes 3 and 4 respectively) prior to mRNA extraction and RT-PCR. Proteins derived from translation of Bethlem myopathy muscle RT-PCR products are shown in lane 5.

protein that was not present in control samples (Fig. 3), indicating the presence of an in-frame premature stop codon within the α1(VI) mRNA. This truncated product was barely detectable in untreated cells (Fig. 3, lane 3) but was present in increased amounts in cells that had been treated with cycloheximide (Fig. 3, lane 4). Importantly, the truncated protein product was also detected in only very low amounts when RNA isolated from a patient muscle biopsy was analysed by the protein truncation test (Fig. 3, lane 5), indicating that nonsense-mediated mRNA decay also occurred in the affected tissue, skeletal muscle. The extremely low levels of mutant mRNA in untreated patient fibroblasts and muscle explain the initial failure to detect the mutation by RT-PCR and direct sequencing (Fig. 4). However, by directly sequencing the RT-PCR product from cycloheximide-treated fibroblasts, we were able to detect the deletion of a single G residue in the mRNA; from the point of the deletion (base 981) the mutant sequence appeared as a less intense band running just below the normal sequence (Fig. 4). This single base deletion was confirmed by cloning and sequencing individual RT-PCR products (data not shown). The codon reading frameshift generated by this mutation results in the appearance of a stop codon 22 amino acids later, at the point predicted by the 37 kDa truncated protein seen by *in vitro* transcription and translation.

The G residue deleted from the α1(VI) mRNA is one of a group of three which are interrupted in the *COL6A1* gene by intron 11 (26,27). To determine the precise nature of the gene mutation, genomic DNA from the 10 normal and nine affected family members was PCR amplified using primers within exons 10 and 13, and directly sequenced. This analysis demonstrated that the mutation is a heterozygous G→A transition at the –1 position of the consensus acceptor splice site of intron 11 (979–1 G→A) (Fig. 5). The mutation was found in all of the affected individuals and was not present in unaffected family members. We found no evidence that the mutation resulted in exon skipping during pre-mRNA splicing by either RT-PCR and direct sequencing, or sequencing of individual cloned RT-PCR products, indicating that the effect of this mutation is not to remove the splice site but to move it 3′ by one base, resulting in the deletion of a G from the mRNA (Fig. 5).

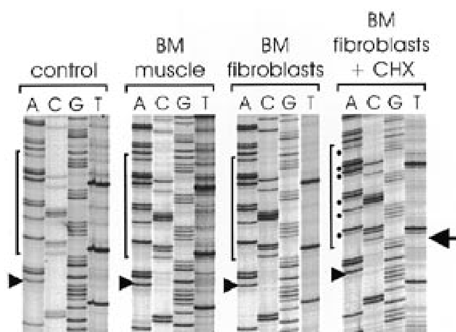

Figure 4. Direct sequencing of control and Bethlem myopathy RT-PCR products. Total RNA was isolated from Bethlem myopathy (BM) fibroblasts which had been treated with 100 μg/ml cycloheximide for 0 and 8 h, from untreated control fibroblasts, and from Bethlem myopathy muscle. Bases 34–2290 of *COL6A1* coding sequences were amplified by RT-PCR and directly sequenced. While the mutant sequence could not be clearly seen in either Bethlem myopathy muscle or untreated fibroblasts, cycloheximide treatment of the patient fibroblasts slowed degradation of the mutant α1(VI) mRNA and allowed detection of the altered sequence. The position of the single G deletion is indicated by the arrow. The resulting abnormal sequence can be seen from the point of the deletion as a shadow band running just below the normal sequence in the cycloheximide-treated Bethlem myopathy fibroblasts. The mutant sequence is highlighted in the A track (●), but is also present in the other lanes. For comparison, the arrowhead indicates that a single band is seen in the A track before the position of the deletion.

α1(VI) haploinsufficiency limits the assembly of functional collagen VI

Each collagen VI triple-helical monomer normally contains one α1(VI), one α2(VI) and one α3(VI) chain (17). Therefore, a

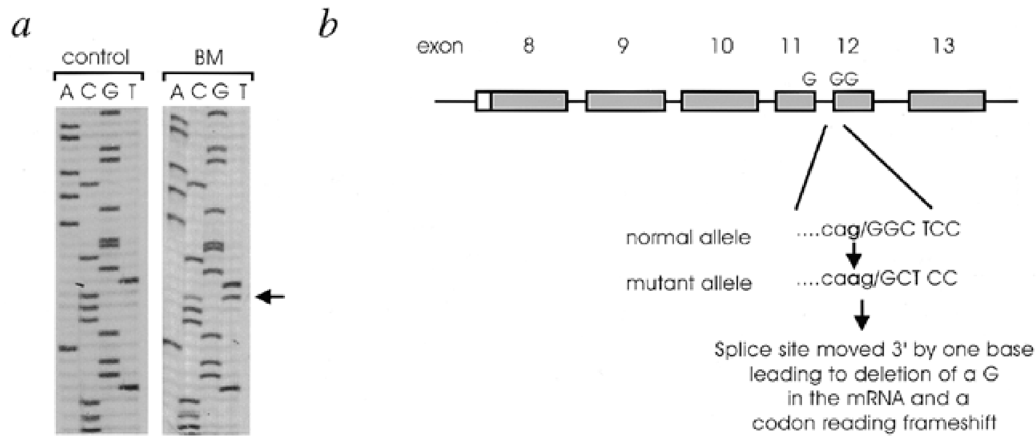

Figure 5. Identification of the mutation in the *COL6A1* gene. Genomic DNA from normal (control) and affected (BM) family members was PCR amplified using primers within exons 10 and 13 of *COL6A1*. The sequence of the non-coding strand is shown in (**a**). The mutation, a heterozygous G→A substitution at the –1 position of the acceptor splice site of intron 11, is indicated by the arrow. The *COL6A1* gene structure in the region surrounding the mutation is depicted in (**b**). Shaded boxes indicate sequences coding for the triple-helical domain, and the mutation and its consequences are shown below.

deficiency of any one of the subunits would be expected to limit the total amount of collagen VI which can be secreted and assembled into extracellular microfibrils. The amount of collagen VI synthesized by fibroblast cultures varies 2- to 3-fold with changes in cell density (28), making it technically difficult to compare collagen VI production accurately in short-term biosynthetic labelling experiments. Using an alternative approach to demonstrate a collagen VI deficiency, control and Bethlem myopathy fibroblasts were pulse-labelled with [^{35}S]methionine for 30 min then chased for up to 4 h in the presence of cold methionine. Intracellular collagen VI was immunoprecipitated and analysed by SDS–PAGE. While the individual collagen VI chains migrated normally under reducing conditions (Fig. 6a), analysis under non-reducing conditions revealed the accumulation in the Bethlem myopathy cells of a disulphide-bonded assembly intermediate (Fig. 6b). This intermediate, which was also present, but at much lower levels, in control cells, migrated faster than a collagen VI triple-helical monomer [α1(VI), α2(VI), α3(VI)], suggesting that it was a normal assembly intermediate composed of an α3(VI) chain disulphide bonded to one other chain, either α1(VI) or α2(VI). Accumulation of this intermediate in the Bethlem myopathy cells over the levels seen in control cells is consistent with the association of α2(VI) and α3(VI) proceeding normally, but further assembly being limited by reduced availability of α1(VI) chains. The α2(VI)–α3(VI) assembly intermediate, containing only two chains, is not triple-helical and is not secreted (data not shown), thus the net effect of α1(VI) haploinsufficiency is assembly and secretion of reduced amounts of collagen VI.

A collagen VI matrix deficiency

To demonstrate further a reduction in collagen VI production, control and Bethlem myopathy fibroblasts were grown for 7 days post-confluence in the continuous presence of sodium ascorbate to allow a collagenous extracellular matrix to be deposited *in vitro* (29). Cell layers were fixed and stained with antibodies against collagen VI and collagen type I (Fig. 7). While collagen I was present at comparable levels in both control and patient extracellular matrices, collagen VI was greatly decreased in the matrix deposited by the Bethlem myopathy fibroblasts, confirming that the *COL6A1* mutation resulted in the production and accumulation of reduced amounts of collagen VI.

DISCUSSION

Mutations which introduce premature translation termination codons are often assumed to result in the production of truncated, functionally defective proteins. However, the primary effect of a premature stop codon is, most commonly, to reduce mRNA stability dramatically, resulting in a virtual absence of the mutant mRNA (22). While the mechanisms responsible for this mRNA decay are not fully understood, four models have been proposed: decay during co-translational export from the nucleus, cytoplasmic decay, nuclear scanning and a cytonuclear feedback mechanism (22). The model which best fits current experimental evidence is nuclear scanning for premature translation termination mutations by an entity that can read codons (25,30). Premature stop codons in the final exon of a gene do not normally reduce mRNA stability, suggesting that down-regulation of nonsense codon-containing mRNA requires at least one spliceable intron downstream of the stop codon, and that recognition of the mutant mRNA may occur in conjunction with mRNA splicing and maturation (30). The observed reversal of this mRNA decay by protein synthesis inhibitors suggests that the nuclear scanner is 'ribosome-like' or that an unstable protein is involved in the down-regulatory mechanism (25). The net effect of a premature stop codon in all but the final exon of most genes is, therefore, protein haploinsufficiency.

The *COL6A1* premature termination mutation identified in this study also resulted in mRNA instability, clearly demonstrated by the 50% reduction in α1(VI) mRNA seen on northern blots, and reinforced by our initial inability to detect the mutant mRNA by RT-PCR and direct sequencing. The intron mutation, 979–1 G→A, within the splice acceptor sequence, moves the 5′ boundary of exon 12 over by one base in the 3′ direction, resulting in the deletion of a G from the mRNA and the appearance of a premature translation termination signal 22 codons later.

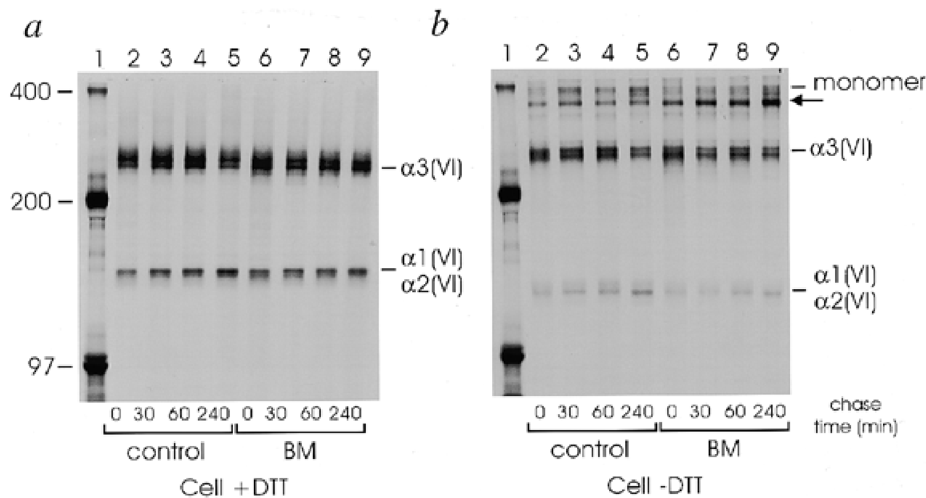

Figure 6. Collagen VI intracellular assembly in Bethlem myopathy fibroblasts. Control and Bethlem myopathy (BM) cultures were pulse-labelled with [^{35}S]methionine for 30 min then chased for up to 240 min in the presence of cold methionine. Intracellular collagen VI was immunoprecipitated and analysed on 5% polyacrylamide gels following reduction with 25 mM DTT (**a**) or under non-reducing conditions (**b**). Molecular weight standards were run in the first lane of each gel. The accumulation in the Bethlem myopathy cells of a disulphide-bonded assembly intermediate is indicated by the arrow in (b). The migration positions of the individual collagen VI subunits α1(VI), α2(VI) and α3(VI) and the triple-helical monomer containing all three chains are shown.

Protection of the unstable abnormal mRNA from nonsense-mediated degradation by treatment with cycloheximide was critical in detecting and characterizing the mutation in this family. The presence of a premature stop codon within α1(VI) mRNA was first detected by the RT-PCR-based protein truncation test. A truncated protein product was clearly evident when cells had been treated with cycloheximide prior to mRNA extraction and RT-PCR, but was barely visible in samples from untreated cells and may have been overlooked. While cycloheximide treatment did not restore mutant mRNA to normal levels, the protection was sufficient to allow detection of the mutant sequence against the background normal sequence by direct sequencing of RT-PCR products. Importantly, this strategy also stabilized the illegitimate nonsense-containing transcripts in transformed lymphoblasts from the patient (J.F. Bateman *et al.*, unpublished data). This novel combination of cycloheximide treatment to stabilize nonsense-containing mRNAs which are transcribed illegitimately by readily accessible cells, and RT-PCR followed by the protein truncation test, should be widely applicable to the diagnosis of genetic diseases such as breast and ovarian cancer, and colon cancer, overcoming current problems with detection due to mutant mRNA instability.

Haploinsufficiency of the α1(VI) subunit, resulting here from the premature termination mutation, and virtually complete breakdown of the mutant mRNA in both fibroblasts and skeletal muscle limited the amount of collagen VI which could be assembled intracellularly into triple-helical molecules, [α1(VI), α2(VI), α3(VI)], and ultimately led to a deficiency in the amount of collagen VI deposited into the extracellular matrix. Neither unassembled α3(VI) chains nor the partially assembled α2(VI)/α3(VI) intermediate were apparent in the medium of the Bethlem myopathy cells, indicating that the excess chains were degraded intracellularly. The degradation pathway was not determined in this study, but other structurally normal unassembled collagen chains, e.g. the proα2(I) in Mov13 cells and osteogenesis imperfecta fibroblasts (31), are degraded by a post-endoplasmic reticulum non-lysosomal mechanism.

The previously characterized collagen VI Bethlem myopathy mutations were glycine substitutions within the triple-helical domain of the α1(VI) and α2(VI) chains (15). Structural mutations of this type in other members of the collagen protein family generally result in relatively severe phenotypes (for reviews, see refs 32,33). The mutant chains assemble intracellularly with normal chains, making the whole triple-helical molecule abnormal, and are secreted and incorporated into the extracellular matrix. The effect of the mutation is amplified further because the mutant molecules are not normally able to form fully functional extracellular assemblies and the presence of even a small number of structurally abnormal molecules can disturb the entire matrix architecture. However, when a reduced amount of collagen is synthesized, the phenotypic consequences are less severe. For example, glycine substitutions in the proα1(I) chain of type I collagen result in severe lethal osteogenesis imperfecta (34), while proα1(I) haploinsufficiency, commonly due to premature termination mutations, leads to mild type I osteogenesis imperfecta (35). While we cannot exclude the possibility that minute amounts of mutant, truncated α1(VI) chains are synthesized by fibroblasts and muscle cells in our family, it is extremely unlikely that mutant chains would be able to associate with normal α2(VI) and α3(VI) and exert a dominant-negative effect. The truncated α1(VI) protein completely lacks not only the C-terminal globular domain, which in the collagens is critical for initial trimerization of the chains and triple helix formation (31,36–38), but also the vast majority of the triple helix, the main structural domain.

The most notable phenotypic difference between the family described here, where reduced production of structurally normal collagen VI is responsible for the Bethlem myopathy, and the families with structural glycine mutations was the reduced incidence of contractures. The presence of contractures does not

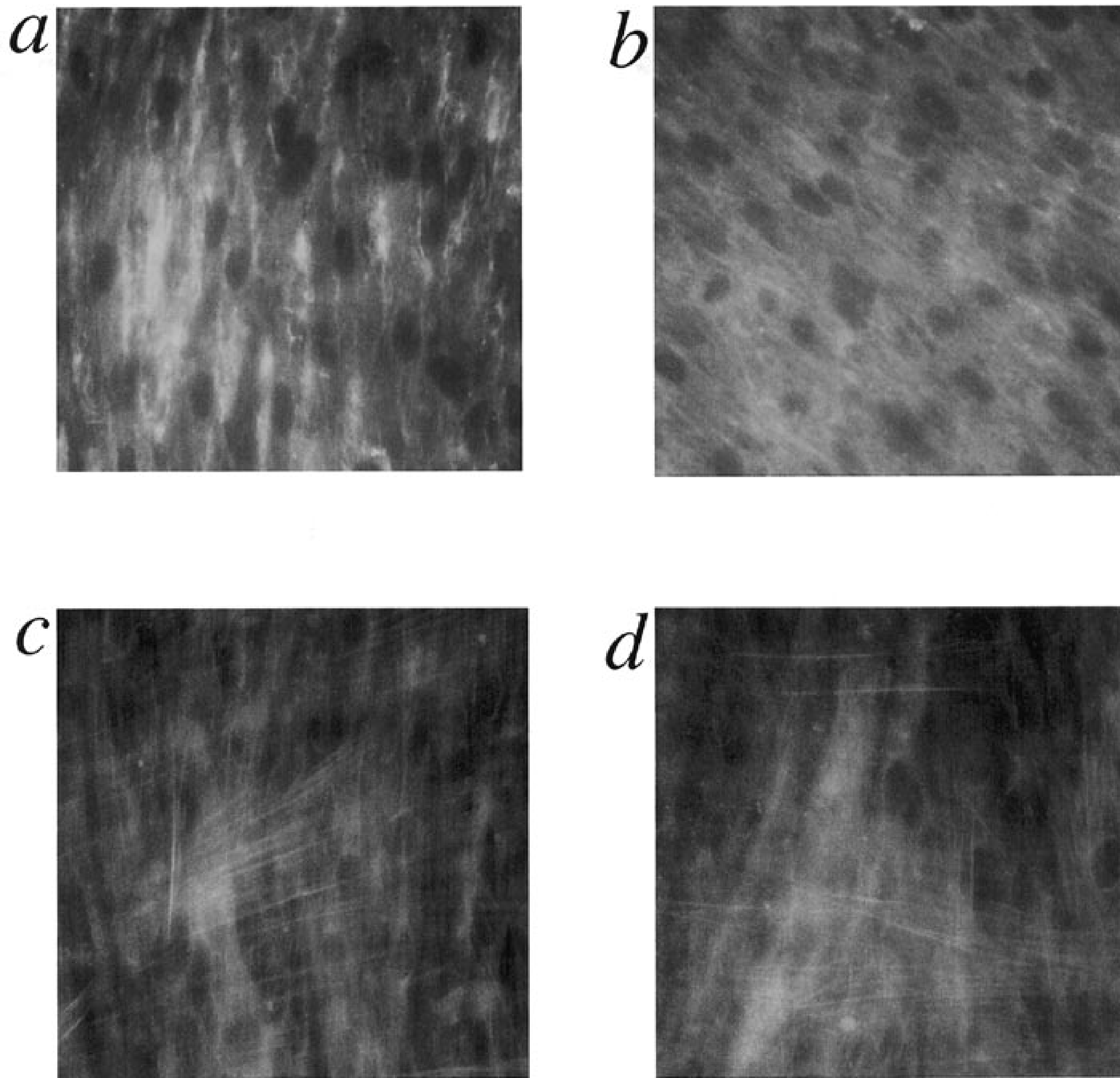

Figure 7. Reduced collagen VI in the *in vitro* accumulated extracellular matrix. Control (**a** and **c**) and Bethlem myopathy fibroblasts (**b** and **d**) were grown for 5 days post-confluence in the presence of 0.25 mM sodium ascorbate then fixed and incubated with antibodies to either collagen VI (a and b) or collagen I (c and d). Bound antibody was detected using fluorescein-conjugated donkey anti-rabbit IgG.

reflect a primary abnormality of the joints, but a shortening of a muscle due to fibrosis or weakness of the antagonist, resulting in limitation of movement of the affected joint (39). The muscular dystrophies show considerable variability both within and between families, and there are examples in a number of Bethlem myopathy families of affected individuals with no apparent contractures (16,40,41). In addition, there seems to be no correlation between the degree of muscle weakness and the severity of contractures within families (16,41,42). It is unclear, therefore, whether the reduced incidence of muscle contractures in our family indicates a less severe phenotype resulting from reduced collagen VI expression rather than a structural mutation, or if it falls within the normal range of phenotypic variability. To resolve this, it will be important to examine collagen VI biosynthesis in patients with structural mutations and determine the fate of the mutant protein. If, for example, the mutations prevent intracellular assembly of dimers and tetramers, triple-helical monomers containing the mutant chain may be degraded intracellularly, again resulting in reduced amounts of normal collagen VI in the extracellular matrix. Interestingly, Jöbsis *et al.* (15) failed to identify a mutation in one of the four Bethlem myopathy families included in their study. While the disease in that family was linked to the *COL6A1–2* cluster, the mutation was not detected by directly sequencing RT-PCR products spanning the entire coding regions of the α1(VI) and α2(VI) chains. It is likely then, that this family may also have a translation termination mutation, and a disease which results from reduced synthesis of collagen VI.

This is the first example of a myopathy caused by reduced synthesis of a structural protein or component of the dystrophin–glycoprotein complex. Premature termination mutations in laminin-2 and α-, β-, γ- and δ-sarcoglycan presumably also result in mRNA instability and protein haploinsufficiency in heterozygotes, since the respective proteins are completely absent in the

homozygous patients (7–9,14,43–45). However, in contrast to α1(VI) haploinsufficiency which results in Bethlem myopathy, in these cases heterozygous carriers show no phenotype. Collagen VI is clearly a critical component of the muscle extracellular matrix, and a reduced amount of structurally normal collagen VI is sufficient to impair skeletal muscle stability and function. While identification of the important cell adhesion or matrix–matrix interactions disturbed by the reduced collagen VI content in Bethlem myopathy skeletal muscle awaits further investigation, collagen VI has been shown by immunoelectron microscopy to be associated intimately with the basement membrane surrounding muscle fibres (19), and to interact *in vitro* with the basement membrane components collagen IV (19) and perlecan (46). These data suggest that one role of collagen VI may be to anchor the muscle cell basement membrane in the surrounding endomyseal extracellular matrix. In addition, collagen VI may be involved in muscle cell adhesion and differentiation via interactions with members of the integrin family of cell surface receptors (47,48) or the membrane-spanning proteoglycan NG2 (49).

MATERIALS AND METHODS

Linkage analysis

Genomic DNA was extracted from leukocytes obtained from nine affected and 10 unaffected family members. Linkage to 21q22.3 was investigated by genotyping the markers *D21S156* and *D21S171*. Two-point and three-point linkage analyses were performed using the MLINK program of the LINKAGE package (50).

RNA isolation, PCR and sequencing

Patient dermal fibroblast cultures were established and maintained as previously described (51). RNA was isolated from cultured fibroblasts using RNeasy™ (Qiagen), and from muscle biopsy by acid/phenol extraction (52). Total RNA (1 µg) was used for reverse transcription with an oligo(dT) primer and reverse transcriptase followed by PCR (GeneAmp®; Perkin-Elmer). The entire *COL6A1* and *COL6A2* coding regions were amplified in a series of overlapping fragments. *COL6A1* cDNA [ATG at base 49 (15)] was amplified in fragments spanning bases 33–924, 730–1882 and 1743–3195. *COL6A2* RT-PCR fragments spanned bases 73–1036 [ATG at base 91 (15)], 798–1816, bases 1617–3206 of the major C-terminal splice variant (α2C2) (53) and bases 1617–2896 of the less common C-terminal splice variant (α2C2a). The minor C-terminal splice variant (α2C2a′) was not analysed. Genomic DNA spanning *COL6A1* exons 10–13 (27) was amplified using primers corresponding to cDNA bases 931–950 and 1110–1029. Amplification products were either cycle sequenced directly (AmpliCycle®; Perkin Elmer), or cloned into a *Sma*I-cut pUC19 vector and cycle sequenced, using internal primers in addition to the original PCR primers, labelling with [α-^{33}P]dATP (2000 Ci/mmol; NEN Research Products).

Northern blotting

Total RNA (5 µg) was separated on denaturing 0.8% agarose gels (54), transferred to nitrocellulose, and fixed at 80°C for 2 h. The filter was hybridized simultaneously to [α-^{32}P]dCTP-labelled α1(VI), α2(VI) and α3(VI) cDNAs [p1, p18, p24 (55)] at 42°C overnight in a buffer containing 50% (v/v) formamide, 5× SSC, 0.02% (w/v) ficoll, 0.02% (w/v) polyvinylpyrrolidone, 0.02% (w/v) bovine serum albumin, 0.1% SDS and 100 µg/ml denatured salmon sperm DNA. The filter was washed twice in 0.1× SSC, 0.1% (w/v) SDS at 65°C for 30 min and exposed to Kodak X-OMAT AR-5 film at –70ºC with an intensifying screen. The hybridization signal was quantified using a phosphorimager (STORM™; Molecular Dynamics).

Protein truncation test

To stabilize mRNAs that may have contained a premature stop codon, patient fibroblasts were incubated with 100 µg/ml cycloheximide (25) for 0, 2 or 8 h prior to RNA extraction. Bases 34–2290 of *COL6A1* coding sequences were amplified by RT-PCR for the protein truncation test using the primers 5′-GCTAATACGACTCATATAGGACAGACCACCATGCCGCAGGCCCGAGACATGAGG-3′ and 5′-CGGGGCTGCAGAGCACGTTGA-3′. The large 5′ primer included the T7 promoter, ribosome-binding sites and an ATG initiation codon (24) in addition to the specific *COL6A1* priming sequence. PCR products were transcribed and translated *in vitro* (TNT™ Coupled Reticulocyte Lysate; Promega) in the presence of L-[^{35}S]methionine (1171 Ci/mmol; ICN Pharmaceuticals). The resultant protein products were reduced with 10 mM dithiothreitol (DTT) and analysed on 14% (w/v) polyacrylamide separating gels with a 4.5% (w/v) stacking gel (51) along with [^{14}C]methylated protein molecular weight markers (Amersham). Radiolabelled proteins were detected by fluorography (51) using Kodak BioMax film.

Biosynthetic labelling and immunoprecipitation

Primary skin fibroblasts were grown to confluency in 10 cm^2 dishes, then incubated overnight in the presence of 0.25 mM sodium ascorbate. Cells were pre-incubated for 30 min in 750 µl of serum-free methionine-free medium containing 0.25 mM sodium ascorbate and then labelled overnight with 100 µCi/ml L-[^{35}S]methionine (TRANS^{35}S-LABEL™, 1032 Ci/mmol; ICN Pharmaceuticals). For the pulse–chase analysis of collagen VI assembly, cells were biosynthetically labelled as above for 30 min, the labelling medium was replaced with serum-free medium containing 20 mM cold methionine and 0.25 mM sodium ascorbate, and the incubation continued for up to 4 h. At the end of the labelling or chase period, the medium was removed to a sterile tube and protease inhibitors added to the following final concentrations; 1 mM phenylmethylsulphonyl fluoride (PMSF), 20 mM *N*-ethylmaleimide (NEM) and 5 mM EDTA. The cell layer was solubilized in 50 mM Tris–HCl, pH 7.5, containing 5 mM EDTA, 150 mM NaCl, 1% NP-40, 1 mM PMSF and 20 mM NEM (cell lysis buffer). Cell lysates and medium samples were clarified by centrifugation, made up to 800 µl by weight with cell lysis buffer, and passed through a 0.45 µm filter (Millipore). Fibronectin, which co-immunoprecipitates with collagen VI and co-migrates on SDS–PAGE with the α3(VI) chain, was removed by gelatin–Sepharose chromatography (Pharmacia Biotech.). The column buffer was 50 mM Tris, pH 7.5 containing 150 mM NaCl, 5 mM EDTA and 0.1% NP-40 (NET buffer). Collagen VI in the column flow-through was immunoprecipitated overnight at 4°C using a specific antibody which recognizes all three collagen VI subunits (56) and 100 µl of 20% protein A–Sepharose (Pharmacia Biotech.). The protein A–Sepharose beads were washed twice with NET buffer, then once with 10 mM Tris–HCl, pH 7.5, 0.1% NP-40 for 30 min each. Immunoprecipitated collagen VI was eluted into gel loading

buffer at 65°C for 15 min and analysed either with or without reduction with 25 mM DTT on 5% (w/v) polyacrylamide gels with a 3.5% (w/v) stacking gel (51).

Composite gel electrophoresis

Assembly of collagen VI triple-helical monomers into disulphide-bonded dimers and tetramers was monitored on composite 2.4% (w/v) acrylamide–0.5% (w/v) agarose gels under non-reducing conditions. Molecular weight standards were the reduced (230 and 400 kDa) and unreduced (900 kDa) forms of laminin (Boehringer Mannheim). Gels were stained overnight with Coomassie brilliant blue R-250 (51) to visualize the molecular weight standards, destained, then dried onto GEL-BOND® film (FMC BioProducts). Radiolabelled proteins were detected using a phosphorimager.

Immunofluorescence

Control and patient fibroblasts were seeded at the same density in glass chamber slides (Nunc) and grown to confluence. Cultures were supplemented daily with 0.25 mM sodium ascorbate for 5 days to allow a collagenous extracellular matrix to be deposited, then fixed with ice-cold methanol for 10 min. The extracellular matrix was incubated with antibodies to collagen VI (Chemicon International) or collagen I (J.F. Bateman *et al.*, unpublished data) for 1 h at room temperature, washed three times with PBS and bound antibody detected using fluorescein-conjugated AffiniPure donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories). Slides were mounted in FluorSave™ (Calbiochem) then viewed and photographed using a Zeiss fluorescence microscope.

ACKNOWLEDGEMENTS

We would like to thank Susanna Freddi for her valuable help with the protein truncation test, and Dr Xenia Dennett (Department of Pathology, University of Melbourne) for generously providing us with archived patient muscle biopsy material. This work was supported by grants from the National Health and Medical Research Council of Australia and the Royal Children's Hospital Research Foundation.

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