Kinked Collagen VI Tetramers and Reduced Microfibril Formation as a Result of Bethlem Myopathy and Introduced Triple Helical Glycine Mutations*

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Mutations in the genes that code for collagen VI subunits, COL6A1, COL6A2, and COL6A3, are the cause of the dominantly inherited disorder, Bethlem myopathy. Glycine mutations that interrupt the Gly-X-Y repetitive amino acid sequence that forms the characteristic collagen triple helix have been defined in four families; however, the effects of these mutations on collagen VI biosynthesis, assembly, and structure have not been determined. In this study, we examined the consequences of Bethlem myopathy triple helical glycine mutations in the \(\alpha1(VI)\) and \(\alpha2(VI)\) chains, as well as engineered \(\alpha3(VI)\) triple helical glycine mutations. Although the Bethlem myopathy and introduced mutations that are toward the N terminus of the triple helix did not measurably affect collagen VI intracellular monomer, dimer, or tetramer assembly, or secretion, the introduced mutation toward the C terminus of the helix severely impaired association of the mutant \(\alpha3(VI)\) chain with \(\alpha1(VI)\) and \(\alpha2(VI)\). Association of the three chains was not completely prevented, however; and some non-disulfide bonded tetramers were secreted. Examination of the secreted Bethlem myopathy and engineered mutant collagen VI by negative staining electron microscopy revealed the striking finding that in all the cell lines a significant proportion of the tetramers contained a kink in the supercoiled triple helical region. Collagen VI tetramers from all of the mutant cell lines also showed a reduced ability to form microfibrils. These results provide the first evidence of the biosynthetic consequences of collagen VI triple helical glycine mutations and indicate that Bethlem myopathy results not only from the synthesis of reduced amounts of structurally normal protein but also from the presence of mutant collagen VI in the extracellular matrix.

**Collagen VI is structurally unique within the collagen family of proteins, forming abundant microfibrils in the extracellular matrix of almost all tissues, including skin, cartilage, cornea, and both skeletal and smooth muscle (for reviews see Refs. 1 and 2). Intracellular assembly of collagen VI is a complex multistep process. Association of the three genetically distinct subunits, \(\alpha1(VI)\), \(\alpha2(VI)\), and \(\alpha3(VI)\), to form a triple helical monomer is followed by antiparallel, staggered assembly into disulfide-bonded dimers, which then align to form tetramers, also stabilized by disulfide bonds. Outside of the cell, tetramers, the secreted form of collagen VI, associate end-to-end to form the characteristic beaded microfibrils.

Bethlem myopathy is a relatively mild, dominantly inherited disorder, characterized by early childhood onset of proximal muscle weakness and wasting and, commonly, joint contractions mainly involving the elbows, ankles, and fingers (3, 4). This disorder was shown to be linked to the COL6A1 and COL6A2 genes on 21q22.3 (5) and in a separate family to the COL6A3 gene at 2q37 (6), and mutations in these collagen VI genes have subsequently been identified in 8 Bethlem myopathy families. The mutations include a single base deletion in the \(\alpha1(VI)\) mRNA that causes a codon reading frameshift and the introduction of a premature stop codon (7), and in two families splice site mutations that lead to the in-frame deletion of \(\alpha1(VI)\) exon 14 encoded sequences (8, 9). Single amino acid substitutions that interrupt the Gly-X-Y amino acid repeat region that forms the characteristic collagen triple helix have been identified in all three genes (10, 11). These glycine mutations are not randomly distributed along the triple helix, but are all within the first 50 amino acids at the N-terminal end of the helix. Only one mutation outside of the triple helix has been reported, a Gly to Glu substitution at amino acid 1679 of the \(\alpha3(VI)\) chain (12). This mutation is in the N2 subdomain, one of the 10 \(\alpha3(VI)\) globular N-terminal subdomains that show homology to the type A domains of von Willebrand factor.

The discovery that heterozygous mutations in any one of the three subunits of collagen VI can cause Bethlem myopathy revealed the critical role of this protein in the muscle extracellular matrix and raised a number of important questions about the consequences of collagen VI mutations on the biosynthesis, assembly, and structure of collagen VI and its interactions in the extracellular matrix. Some of the Bethlem myopathy mutations, including a premature stop codon in the \(\alpha1(VI)\) chain and the deletion of \(\alpha1(VI)\) exon 14, result in the secretion of reduced amounts of structurally normal collagen VI (7, 9); however, the effects of single amino acid substitutions on collagen VI structure and function are unknown. In this study, we examine the biosynthetic and structural consequences of Bethlem myopathy and engineered glycine mutations within the triple helical domains of the \(\alpha1(VI)\), \(\alpha2(VI)\), and \(\alpha3(VI)\) chains.
The Bethlem myopathy mutations and the engineered triple helical glycine mutations. Diagram of collagen VI α3(VI), α2(VI), and α3(VI) chains showing the triple helix and numbered C- and N-terminal subdomains. The shaded type A subdomains, N10, N9, and N7 in α3(VI) and C2 in α2(VI) undergo alternative splicing. The α(VI) and α2(VI) Bethlem myopathy mutations and the engineered α3(VI) mutations are shown below each chain. Amino acids are numbered from the first glycine of the triple helix.

EXPERIMENTAL PROCEDURES

Bethlem Myopathy Patients and Mutation Nomenclature—Detailed clinical descriptions of the two Bethlem myopathy families included in this study have been published previously (4). The mutations in these families are heterozygous point mutations leading to α1(VI) G286V and α2(VI) G250S substitutions (11). Both mutations are within the collagen VI triple helical domain. To allow the positions of these mutations to be readily compared with the introduced α3(VI) mutations also described in this study, we refer to these mutations as α1(VI) G49V and α2(VI) G168S, respectively, to indicate the position of the mutations relative to the start of the triple helical domain (Fig. 1).

Site-directed Mutagenesis of the α3(VI) Chain—Two α2(VI) cDNA expression constructs containing glycine substitutions within the triple helical domain were produced using strand overlap extension PCR (13). To introduce a Gly to Ala mutation at residue 49 of the triple helix, bases 5708–5895 (domains N9-C5, ATG at base 526 (14) and 5875–6098 were amplified by PCR in separate reactions using the α3(VI) N6-C5 plasmid (15) as the template. The overlapping primers each contained two nucleotide substitutions that resulted in the G49A mutation (GCC-GCA). These primary amplification products were gel-purified, and 100 ng of each was used as the template in a second round of overlapping PCR. The resulting PCR product (bases 5708–6098) was digested with SacII and XhoI and used to replace the corresponding fragment of a 2.2-kilobase α3(VI) SacII-BstEII 1107I subclone. Similarly, a Gly to Val mutation at residue 301 of the triple helix was introduced by PCR amplification of bases 6204–6651 and 6631–7422 in which the overlapping primers contained the G301V (GCC-GGT) change. The second round overlapping PCR product (bases 6204–7422) was digested with MluNI and ligated into the 2.2-kilobase SacII-BstEII 1107I subclone. Finally, the 1.7-kilobase SacII-Eco47III fragment of the α3(VI) N6-C5 plasmid (15) was replaced with the corresponding mutant fragments to produce the expression plasmids α3(VI) N6-C5 G49A and α3(VI) N6-C5 G301V. To confirm the sequence changes and ensure that no errors had been introduced during PCR and cloning, the mutant constructs were transcribed and translated in vitro (TNTβ, Promega), and the regions generated by PCR were sequenced (Ampliclone™, PerkinElmer Life Sciences) (data not shown).

Cell Culture and Transfection—Human dermal fibroblasts were established from skin biopsies (11), and the human osteosarcoma cell line, SaOS-2 (ATCC HTB-85), was obtained from American Type Culture Collection. Cell cultures were maintained in Dulbecco’s modified Eagle’s medium containing 10% (v/v) fetal calf serum as described previously (16). SaOS-2 cells were transfected with the α3(VI) cDNA expression constructs using LipofectAMINE reagent (Invitrogen) according to the manufacturer’s protocol. Stably transfected cells were selected in growth medium containing 500 μg/ml G418 (Invitrogen), and individual G418-resistant colonies were isolated and expanded into cell lines. G418 was removed from the culture medium after the fourth passage.

Collagen VI Biosynthetic Labeling and Analysis—Primary skin fibroblasts and SaOS-2 cells were grown to confluence in 10-cm² dishes, incubated overnight in the presence of 0.25 mM sodium ascorbate, and then biosynthetically labeled for 18 h with 100 μCi/ml [35S]methionine (Trans™S-label 1032 Ci/mmol, ICN Pharmaceuticals, Inc.) in 750 μl of methionine-free and serum-free Dulbecco’s modified Eagle’s medium containing 0.25 mM sodium ascorbate. The medium was removed to a sterile tube, and protease inhibitors were added to the following final concentrations: 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, 20 mM N-ethylmaleimide, and 5 mM EDTA. The cell layer was solubilized for 30 min in 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, and 20 mM N-ethylmaleimide (cell lysis buffer). Cell lysates and medium samples were clarified by centrifugation, made up to 800 μl with cell lysis buffer, and passed through a 0.45-μm filter (Millipore). Fibronectin, which co-immunoprecipitates with collagen VI and co-migrates with the α3(VI) chain on SDS-polyacrylamide gels, was removed by gelatin-Sepharose chromatography (Amersham Biosciences). The column buffer was 50 mM Tris/HCl, pH 7.5, containing 150 mM NaCl, 5 mM EDTA, and 0.1% Nonidet P-40 (NET buffer). Collagen VI in the column flow-through was immunoprecipitated overnight at 4 °C using the α3(VI) monoclonal antibody 3C4 (17) and 100 μl of 20% protein A-Sepharose (Amersham Biosciences). The protein A-Sepharose beads were washed twice with NET buffer and then once with 10 mM Tris/HCl, pH 7.5, 0.1% Nonidet P-40 for 30 min each. Immunoprecipitated collagen VI was eluted into gel loading buffer at 65 °C for 15 min and analyzed following reduction with 25 mM dithiothreitol by SDS-PAGE on 5% (w/v) polyacrylamide gels. Collagen VI triple helical monomers, dimers, and tetramers were analyzed on 2.4% (w/v) agarose composite gels under non-reducing conditions as described previously (9, 15). Radioactively labeled proteins were detected by fluorography (16) or imaged using a PhosphorImager (Molecular Dynamics, STORM™).

Negative Staining Electron Microscopy—Confluent human fibroblasts and SaOS-2 cultures in 80-cm² flasks were incubated for 18 h in 10 ml of serum-free medium containing 0.25 mM sodium ascorbate. The medium was collected; the protease inhibitors described earlier were added, and sodium azide was included at a final concentration of 0.1% (v/v). Culture medium was clarified by centrifugation, adsorbed onto carbon-coated grids for 1 min, washed with water, and stained with 0.75% uranyl formate. The grids were rendered hydrophobic by glow discharge in air, and samples were observed in a Jeol 1200 EX electron microscope operated at 60 kV accelerating voltage.

Indirect Immunofluorescence—Human fibroblasts and SaOS-2 cells were grown to confluence in 8-well chamber glass slides (Nunc) and then supplemented daily for up to 15 days with 0.25 mM sodium ascorbate. Cell layers were washed with PBS, fixed with 3.7% (v/v) formaldehyde at room temperature for 10 min, and then air-dried. Slides were preincubated with 5% (v/v) fetal calf serum in PBS for 1 h at room temperature, and then the primary antibody (either the monoclonal antibody 3C4 in PBS or PBS alone for 1 h. Bound antibody was detected using fluorescein isothiocyanate-conjugated sheep anti-mouse Ig (Silenus). Slides were mounted in FluorSave™ reagent (Calbiochem) and then observed using a Zeiss fluorescence microscope.

Gel Filtration Chromatography—SaOS-2 cells were grown to confluence in 175-cm² flasks and then incubated overnight in 10 ml of serum-free medium containing 0.25 mM sodium ascorbate. The medium was collected, and 1 ml 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride and 20 mM N-ethylmaleimide were added. Samples were clarified by centrifugation and then dialyzed overnight against 50 mM Tris/HCl, pH 7.5, containing 150 mM NaCl. Aliquots of the medium (500 μl) were either applied directly to a Superose 6 HR 10/30 column (Amersham Biosciences) or first digested for 2 h at 37 °C with 20 μg of collagenase (Warthington, CLS-2) in the presence of 10 mM CaCl₂. The digestion was terminated by the addition of 20 mM EDTA. The Superose 6 column was run at 0.5 ml/min in 50 mM Tris/HCl, pH 7.5, containing 150 mM NaCl, and 0.5-m1 fractions were collected. Column fractions were dotted onto nitrocellulose membrane and collagen VI α3(VI) chains detected using the monoclonal antibody 3C4. Bound antibody was detected using an anti-mouse horseradish peroxidase-conjugated antibody and an enhanced chemiluminescence kit (ECL Plus, Amersham Biosciences). The x-ray films were scanned (Bio-Rad, GS-800 Calibrated Densitometer) to determine the relative amount of collagen VI in each fraction.

RESULTS

Collagen VI Intracellular Assembly and Secretion Are Not Affected by Heterozygous Bethlem Myopathy Triple Helical Glycine Mutations—To examine the biosynthetic consequences of

1 The abbreviation used is: PBS, phosphate-buffered saline.
Kinked Collagen VI Tetramers

Fig. 2. Electrophoretic analysis of collagen VI produced by Bethlem myopathy fibroblasts. Control fibroblasts, fibroblasts from two Bethlem myopathy patients with an α1(VI) G49A mutation, and a patient with an α2(VI) G16S mutation were biosynthetically labeled overnight with [35S]methionine, and the collagen VI in the cell (C) and medium (M) fractions was immunoprecipitated. a, samples analyzed under reducing conditions on a 5% polyacrylamide gel. The migration positions of the individual collagen VI subunits α1(VI), α2(VI), and α3(VI) are indicated on the right. b, samples analyzed without reduction on a composite 0.5% (w/v) agarose, 2.5% acrylamide (w/v) gel. The collagen VI disulfide-bonded tetramers are indicated on the right.

Fig. 3. Collagen VI in the in vitro accumulated extracellular matrix of Bethlem myopathy fibroblasts. Human fibroblasts from a control (a), two patients with an α1(VI) G49V mutation (b and c), and a patient with an α2(VI) G16S mutation (d) were grown for 8 days post-confluence in the presence of 0.25 mM sodium ascorbate and stained with a collagen VI antibody. Bound antibody was detected with fluorescein isothiocyanate-conjugated sheep anti-mouse Ig. Images are ×40.

disease-causing glycine mutations within the triple helical domain of collagen VI, control fibroblasts and fibroblasts from patients with an α1(VI) G49V and an α2(VI) G16S mutation were labeled overnight with [35S]methionine. Collagen VI in the cell and medium fractions were immunoprecipitated and analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions. All of the collagen VI subunits, α1(VI), α2(VI), and α3(VI), produced by the Bethlem myopathy fibroblasts migrated normally and were efficiently secreted into the medium (Fig. 2a, lanes 1–6). Total production of collagen VI was comparable in control and patient cultures (Fig. 2a) suggesting that the mutant chains were able to fold correctly and were not targeted for intracellular degradation. The normal pathway of collagen VI intracellular assembly involves the formation of triple helical monomers containing all three chains, then disulfide-bonded dimers (6 chains), and then tetramers (12 chains) which are secreted from the cell. Analysis of collagen VI on non-reducing gels demonstrated that in both control and Bethlem myopathy cultures, collagen VI tetramers were the major secreted form (Fig. 2b, lanes 2, 4, 6, and 8), and collagen VI monomers and dimers did not accumulate within the Bethlem myopathy cells (Fig. 2b, lanes 1, 3, and 5). Taken together, these data indicate that the triple helical glycine mutations do not disturb chain association and folding of the triple helix, the formation of disulfide-bonded dimers and tetramers, or secretion of the mutant-containing molecules from the cell.

To examine the effect of these mutations on the formation of a collagen VI extracellular matrix, fibroblasts were grown for 8 days post-confluence in the presence of sodium ascorbate, stained with a collagen VI antibody, and examined by fluorescence microscopy. Control and Bethlem myopathy cultures all expressed and secreted the collagen VI cDNA expression constructs were produced by site-directed mutagenesis and stably expressed in SaOS-2 cells. SaOS-2 cells transcribe α1(VI) and α2(VI) mRNAs at levels comparable with that of skin fibroblasts but do not synthesize α3(VI) mRNA and produce no stable collagen VI protein (15). Normal collagen VI biosynthesis and matrix deposition can be restored in these cells by stable transfection with an α3(VI) cDNA construct encoding protein domains N6-C5 (15), thus making this cell line an ideal model system for examining the effect of mutations in the α3(VI) chain in the absence of endogenous normal α3(VI) protein synthesis. The first α3(VI) expression construct contained a Gly to Ala mutation at amino acid 49 of the triple helix (α3(VI) G49A and was designed to mimic the reported Bethlem myopathy glycine mutations that are all localized toward the N terminus of the triple helix (Fig. 1) (10, 11). In the second construct the mutation was toward the C-terminal end of the triple helix, a Gly to Val substitution at amino acid 301 (α3(VI) G301V) and was designed to mimic the reported Bethlem myopathy glycine mutations that are all localized toward the N terminus of the triple helix (Fig. 1) (10, 11). In the second construct the mutation was toward the C-terminal end of the triple helix, a Gly to Val substitution at amino acid 301 (α3(VI) G301V). Both constructs encode α3(VI) protein domains N6-C5. Stably transfected clonal SaOS-2 cell lines expressing the control construct α3(VI) N6-C5 and the two mutant constructs α3(VI) N6-C5 G49A and α3(VI) N6-C5 G301V were biosynthetically labeled overnight, and collagen VI in the cell and medium fractions were immunoprecipitated with the α3(VI)-specific antibody 3C4 and analyzed under reducing conditions on 5% polyacrylamide gels as before. In both control cells and cells expressing the α3(VI) G49A chain, α1(VI) and α2(VI) were co-immunoprecipitated with the α3(VI) chain, and all three chains were efficiently secreted (Fig. 4a, lanes 1–4). Analysis under non-reducing conditions clearly demonstrated the presence of collagen VI tetramers in the cell and medium of the α3(VI) G49A cells (Fig. 4b, lanes 3 and 4), indicating that intracellular assembly and secretion proceed normally, even when all of the collagen VI monomers contain an α3(VI) chain with an N-terminal triple helical glycine mutation. In contrast, co-immunoprecipitation of α1(VI) and α2(VI) chains was poor in both the cell and medium of α3(VI) G301V-expressing cells despite an abundance of α3(VI) (Fig. 4a, lanes 5 and 6), suggesting that this C-terminal helical mutation severely disrupted assembly of the mutant α3(VI) chain with endogenous α1(VI) and α2(VI). The major protein band seen under non-reducing conditions was the α3(VI) chain (Fig. 4b, lanes 5 and 6), consistent with the large excess of α3(VI) seen under reducing conditions (Fig. 4a, lanes 5 and 6). Disulfide bonded monomers and dimers were present in both the cell and medium.
fractions, but disulfide-bonded tetramers were not observed (Fig. 4b, lanes 5 and 6). This was a consistent finding in three separate experiments.

Previous studies (9) in both human fibroblasts and SaOS-2 cells have shown that when collagen VI monomers contain a deletion in the triple helix that prevents dimer and tetramer formation, the monomers are retained within the cell and are not secreted. The presence of disulfide-bonded monomers and dimers in the medium of α3(VI) G301V-expressing cells raised the possibility that tetramers were indeed assembled within these cells, allowing them to be secreted, but these tetramers were not disulfide-bonded and so dissociated under the denaturing gel electrophoresis conditions. To explore this possibility, the medium from control cells and cells expressing α3(VI) G49A and α3(VI) G301V chains was analyzed by gel filtration chromatography under non-denaturing and non-reducing conditions. Column fractions were dotted onto nitrocellulose and α3(VI) chains detected using the 3C4 antibody. The medium from all three cell lines contained high molecular weight α3(VI)-reactive material that eluted in the void volume (Fig. 5). An additional, later eluting peak was resistant to bacterial collagenase digestion (19, 20). As expected, the free α3(VI) chains seen in undigested medium from control and α3(VI) G301V cells were removed by collagenase digestion (Fig. 5, a and c). The collagen VI eluting in the void volume, however, was resistant to collagenase, not only in the control and α3(VI) G49A medium but also in the α3(VI) G301V medium (Fig. 5), suggesting that tetramers were assembled and secreted by all three cell lines. Together with the electrophoretic data (Fig. 4), this led to the conclusion that the α3(VI) G301V mutation affected collagen VI intracellular assembly in two ways. First, assembly of the mutant chains with α1(VI) and α2(VI) was severely compromised but not completely prevented. Second, whereas the mutation did not prevent the formation of tetramers, the disulfide bonds that normally stabilize the tetramers were unable to form.

To determine whether tetramers containing the α3(VI) G49A and G301V mutations were able to form a collagen VI extracellular matrix, stably transfected SaOS-2 cells were grown for 15 days post-confluence in the presence of sodium ascorbate and stained with the 3C4 antibody. A collagen VI extracellular matrix that was uniformly distributed over the cells was seen in both control and α3(VI) G49A cultures (Fig. 6, a and b), suggesting that the α3(VI) G49A mutation did not prevent the end-to-end assembly of tetramers to form microfibrils. Extracellular collagen VI staining was also seen in α3(VI) G301V cultures; however, the staining was not evenly distributed over the cells, and only small localized patches were observed, one of which is shown (Fig. 6c). This uneven, patchy matrix staining is most likely a consequence of the small amount of collagen VI that is assembled and secreted by the α3(VI) G301V cells, and its presence suggests that the disulfide-bonded tetramers secreted by these cells are able to assemble into microfibrils.

Collagen VI Ultrastructure and Microfibril Formation—To
visualize the collagen VI tetramers secreted by the SaOS-2 α3(VI) G301V cells, and to quantify microfibril formation, medium from the transfected SaOS-2 cells and control and Bethlem myopathy fibroblasts was examined by negative staining electron microscopy. Although this analysis confirmed the presence of collagen VI tetramers in the medium of α3(VI) G301V cells, the most striking finding was that a significant proportion of the usually linear tetramers contained a kink, approximately one-third of the way along the supercoiled triple helical domain (Fig. 7G). Examination of SaOS-2 α3(VI) G49A and the Bethlem myopathy culture medium demonstrated that these cells also produced kinked tetramers (Fig. 7, A and C–F). Kinked tetramers were not seen in either control fibroblasts or control SaOS-2 cells. The kinks were approximately the same distance along the supercoiled domain in all of the mutant cell lines, and the position of the kink did not correlate with the position of the underlying mutations. This suggested that the mutations perturbed the structure of the triple helix, and as a consequence, supercoiling of the triple helices during dimer and tetramer formation was abnormal, producing a kink in some of the molecules, possibly at a site of relatively high flexibility.

Microfibril formation in the Bethlem myopathy and transfected SaOS-2 cells was quantified by analysis of a large number of micrograph fields (Fig. 8). The occurrence of “microfibrils” containing 1–7 tetramers is shown as a percentage of the total number of microfibrils. In control fibroblasts around 20% of the microfibrils were single tetramers and microfibrils containing up to 7 tetramers were seen (Fig. 8a). The medium from Bethlem myopathy cells, however, contained a larger proportion of single tetramers, accounting for around 40% of the microfibrils, and microfibrils containing more than 5 tetramers were not observed (Fig. 8a), indicating that the triple helical glycine mutations led to a reduction in the efficiency of end-to-end association of tetramers. Assembly of tetramers into microfibrils was similarly reduced in SaOS-2 cells transfected with the two mutant α3(VI) constructs (Fig. 8b). When compared with the control, the G49A and G301V medium contained a larger proportion of single tetramers and fewer large microfibrils.

The reduced ability of tetramers produced by the Bethlem myopathy cells to form microfibrils was not detected by visual comparison of immunofluorescent staining of the collagen VI matrix deposited by control and Bethlem myopathy cultures (Fig. 3). The fibroblast extracellular matrices were therefore examined in more detail by transmission electron microscopy (data not shown). Morphometric analysis revealed that 68% of the surface area of the control culture was covered with collagen VI microfibrils, compared with 43, 41, and 45% of the surface area of the two α1(VI) G49V and the α2(VI) G16S cultures, respectively. This ~35% reduction in the amount of collagen VI in the matrix thus confirmed that microfibril formation was less efficient in the Bethlem myopathy cultures.

**DISCUSSION**

The study of disease causing mutations has proven to be critical in dissecting developmental events, biochemical pathways, and the roles of many structural proteins. However, characterization of the mutations is only the first step toward a comprehensive understanding of disease pathology. Although glycine mutations within the triple helical domain of the three collagen VI α-chains are known to cause Bethlem myopathy, the effect of these mutations on collagen VI biosynthesis, assembly, and structure had not been determined. In this study, we have examined the consequences of naturally occurring and introduced collagen VI triple helical glycine mutations. Biosynthetic analyses of the collagen VI produced by fibroblasts from Bethlem myopathy patients with a Gly to Val mutation at amino acid 49 of the α1(VI) triple helical domain and a Gly to Ser substitution at amino acid 16 of the α2(VI) triple helix demonstrated that these mutations did not prevent chain association and folding of the triple helix, the formation of disulfide-bonded dimers and tetramers, or secretion of the mutant-containing molecules from the cell. Similarly, an engineered Gly to Ala mutation at amino acid 49 of the α3(VI) triple helix
did not affect intracellular assembly and secretion in stably transfected SaOS-2 cells. Ultrastructural analysis of the collagen VI secreted by Bethlem myopathy fibroblasts and the transfected SaOS-2 a3(VI) control, a3(VI) G49A, and a3(VI) G301V constructs (b) was visualized by negative staining electron microscopy, and the ability of the tetramers to associate end-to-end was quantitated. The occurrence of microfibrils containing 1–7 tetramers is shown as a percentage of the total number of microfibrils.

Although the Bethlem myopathy and introduced glycine mutations that are toward the N terminus of the triple helix do not measurably affect collagen VI intracellular assembly, the engineered glycine mutation toward the C-terminal end of the helix has provided important new information about the requirements for folding of the triple helix and the subsequent formation of dimers and tetramers. The requirements for triple helix folding have been most thoroughly studied in the fibrillar collagens. The initial chain association event, involving inter- and intrachain interactions between the C-terminal globular domains (21), is followed by nucleation of triple helix folding. Nucleation requires proline hydroxylation (21) and at least two consecutive C-terminal Gly-X-Y triplets (22). This is presumably because the Y-position amino acid that confers the most stability to the collagen triple helical structure is hydroxyproline (23). By analogy, the collagen VI C-terminal globular domains have been proposed to be critical for chain association; however, this has not yet been shown experimentally. Our data demonstrating glycine mutations toward the N-terminal end of the triple helix do not affect collagen VI assembly but a glycine mutation toward the C terminus severely impairs association of the three chains can be explained if the collagen VI triple helix is mutated to a valine in this study, is shown in bold. The interruption to the triple helix that corresponds to the position of the kink in the supercoiled triple helical region of the tetramer is boxed. b, schematic drawing of a collagen VI dimer. Two collagen VI monomers (gray and black) are associated in an anti-parallel fashion with a stagger of 30 nm and an overlap of 75 nm. The lines represent the triple helical domains, and the ovals represent globular domains at the C-terminal (COOH) and N-terminal (NH2) ends of the triple helix. The triple helical cysteines (C) important for dimer (a1(VI) or a2(VI) Cys-89)) and tetramer (a3(VI) Cys-50) stabilization are indicated. The positions of the interruptions to the Gly-X-Y amino acid sequence of the monomers that allow supercoiling of the triple helices are indicated by the black and gray arrows. The asterisks indicate the interruption that corresponds to the position of the kinks. The locations of the Bethlem myopathy and introduced mutations in the two monomers are shown by the triangles below the dimer.

Fig. 8. Quantitative analysis of collagen VI tetramer-tetramer association. Collagen VI secreted into the medium of control and Bethlem myopathy fibroblasts (a) and SaOS-2 cells transfected with the a3(VI) control, a3(VI) G49A, and a3(VI) G301V constructs (b) was visualized by negative staining electron microscopy, and the ability of the tetramers to associate end-to-end was quantitated. The occurrence of microfibrils containing 1–7 tetramers is shown as a percentage of the total number of microfibrils.

Fig. 9. Collagen VI sequences and domains involved in intracellular assembly. a, amino acid sequence of the C-terminal triple helical domains of the a1(VI), a2(VI), and a3(VI) chains. The sequences are shown aligned from the C terminus of the triple helix. Amino acids are numbered from the N-terminal end of the helix and are shown on the right. To maintain alignment of the glycine residues (shaded), a1(VI) Asn-304 is shown above the a1(VI) sequence. Glycine 301 of the a3(VI) triple helix, which was mutated to a valine in this study, is shown in bold. The interruption to the triple helix that corresponds to the position of the kink in the supercoiled triple helical region of the tetramer is boxed. b, schematic drawing of a collagen VI dimer. Two collagen VI monomers (gray and black) are associated in an antiparallel fashion with a stagger of 30 nm and an overlap of 75 nm. The lines represent the triple helical domains, and the ovals represent globular domains at the C-terminal (COOH) and N-terminal (NH2) ends of the triple helix. The triple helical cysteines (C) important for dimer (a1(VI) or a2(VI) Cys-89)) and tetramer (a3(VI) Cys-50) stabilization are indicated. The positions of the interruptions to the Gly-X-Y amino acid sequence of the monomers that allow supercoiling of the triple helices are indicated by the black and gray arrows. The asterisks indicate the interruption that corresponds to the position of the kinks. The locations of the Bethlem myopathy and introduced mutations in the two monomers are shown by the triangles below the dimer.
and this must be sufficient to nucleate folding in this collagen type. Following the nine most C-terminal Gly-X-Y triplets, both α1(VI) and α2(VI) contain interruptions to the triple helical sequence (Fig. 9a), requiring that the triple helix renucleate. This occurs despite a relatively low proportion of Y-position proline residues following the interruption. The introduced α3(VI) G301V mutations extends this interruption to the triple helix so that it now involves three consecutive Gly-X-Y triplets. Severely impaired assembly of this mutant α3(VI)/H9251X-Y occurs despite a relatively low proportion of sequence (Fig. 9a), requiring that the triple helix renucleate. The introduced proline residues following the interruption. The introduced α3(VI)/H9251X-Y type. Following the nine most C-terminal Gly-...
