Collagen VI Microfibril Formation Is Abolished by an α2(VI) von Willebrand Factor Type A Domain Mutation in a Patient with Ullrich Congenital Muscular Dystrophy*

Received for publication, June 7, 2010, and in revised form, July 22, 2010 Published, JBC Papers in Press, August 21, 2010, DOI 10.1074/jbc.M110.152520

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Collagen VI is an extracellular protein that most often contains the three genetically distinct polypeptide chains, α1(VI), α2(VI), and α3(VI), although three recently identified chains, α4(VI), α5(VI), and α6(VI), may replace α3(VI) in some situations. Each chain has a triple helix flanked by N- and C-terminal globular domains that share homology with the von Willebrand factor type A (VWA) domains. During biosynthesis, the three chains come together to form triple helical monomers, which then assemble into dimers and tetramers. Tetramers are secreted from the cell and align end-to-end to form microfibrils. The precise molecular mechanisms responsible for assembly are unclear. Mutations in the three collagen VI genes can disrupt collagen VI biosynthesis and matrix organization and are the cause of the inherited disorders Bethlem myopathy and Ullrich congenital muscular dystrophy. We have identified a Ullrich congenital muscular dystrophy patient with compound heterozygous mutations in α2(VI). The first mutation causes skipping of exon 24, and the mRNA is degraded by nonsense-mediated decay. The second mutation is a two-amino acid deletion in the C1 VWA domain. Recombinant C1 domains containing the deletion are insoluble and retained intracellularly, indicating that the mutation has detrimental effects on domain folding and structure. Despite this, mutant α2(VI) chains retain the ability to associate into monomers, dimers, and tetramers. However, we show that secreted mutant tetramers containing structurally abnormal C1 VWA domains are unable to associate further into microfibrils, directly demonstrating the critical importance of a correctly folded α2(VI) C1 domain in microfibril formation.

Collagen VI is a microfibrillar protein broadly expressed in connective tissues. It is composed of three genetically distinct polypeptide chains, α1(VI), α2(VI), and, most often, α3(VI), encoded by the genes COL6A1, COL6A2, and COL6A3, respectively. Three recently identified chains, α4(VI), α5(VI), and α6(VI), may replace α3(VI) in some situations (1, 2). Each chain consists of a short Gly-X-Y triple helical sequence (3) flanked on either side by a number of globular domains, the majority of which share homology with the von Willebrand factor type A (VWA) domains (4–6). Each chain has two C-terminal VWA domains (C1 and C2). In addition, the α3(VI) chain has three C-terminal domains that are not found in α1(VI) or α2(VI); C3 is proline-rich and similar to some salivary proteins, C4 has homology to fibronectin type III repeats, and C5 shows homology to the Kunitz family of serine protease inhibitors (6). The α1(VI) and α2(VI) chains each have one N-terminal VWA domain (N1), whereas the α3(VI) chain has up to 10 N-terminal VWA domains (N1–N10), some of which are subject to alternative splicing (7, 8).

Collagen VI assembly begins with the intracellular association of α1(VI), α2(VI), and α3(VI) chains to form a monomer that consists of a 105-nm triple helical segment flanked on either side by the N- and C-terminal globular domains (9). It is predicted that this initial chain association is mediated by the C1 domains (10, 11). Two monomers then align in anti-parallel fashion, with a 75-nm overlap, to form a dimer that is stabilized by disulfide bonds (3, 9). Tetramers, also stabilized by disulfide bonds, are formed via the lateral association of two dimers with their ends in register and are secreted into the extracellular matrix. Here, tetramers align end-to-end to form microfibrils (9). Electron microscopy has revealed that collagen VI microfibrils consist of repeating units of triple helical segments, joined by large “beaded” regions made up of the overlapping N- and C-terminal VWA domains of adjacent tetramers (9, 12, 13).

Although the basic mechanisms of collagen VI assembly are understood, the full extent of interactions that facilitate monomer, dimer, tetramer, and microfibril assembly is not yet understood. One useful method for defining regions of the collagen VI chains involved in protein assembly is to study collagen VI

4 The abbreviations used are: VWA, von Willebrand factor type A; UCMD, Ullrich congenital muscular dystrophy; AEBSF, 4-(2-aminoethyl)-benzenesulfonfyl fluoride hydrochloride; CHAPS, 3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; MIDAS, metal ion-dependent adhesion site.
biosynthesis in muscular dystrophy patients with known mutations in the collagen VI genes. Mutations in all three collagen VI genes are the cause of Bethlem myopathy and Ullrich congenital muscular dystrophy (UCMD). These disorders show both dominant and recessive inheritance and form a spectrum of clinical severity from mild weakening of the proximal muscles and contractures of the ankles, elbows, and interphalangeal joints in Bethlem myopathy to a severe and progressive phenotypic course characterized by congenital muscle weakness, proximal joint contractures, hyperextensibility of the distal joints, and severe scoliosis in UCMD (14–16).

The majority of known collagen VI mutations are clustered in the N-terminal region of the triple helix (16). Studying collagen VI biosynthesis in patients with these mutations has proved particularly useful in dissecting the regions of the triple helix involved in collagen VI assembly; some in-frame N-terminal triple helical deletions remove cysteine residues that are critical for stabilizing dimers (17–19), whereas other in-frame deletions or glycine substitutions in the Gly-X-Y repeats disrupt the structure of a region of the triple helix involved in tetramer and microfibril formation (20). Amino acid substitutions and in-frame deletions in the collagen VI VWA domains have also been identified in Bethlem myopathy and UCMD patients. Bio-synthetic analyses in a small number of these patients have revealed that the pathogenic consequences of the collagen VI VWA domain mutations are varied, with some preventing incorporation of mutant chains into monomers, whereas others have no obvious effect on protein assembly (17, 21–23).

We have identified a UCMD patient, UCMD21, with compound heterozygous α2(VI) mutations. The first mutation results in the introduction of a premature termination codon and degradation of the mRNA from the affected allele. The second mutation is a two-amino acid deletion in the N-terminal region of the α2(VI) C1 VWA domain. Because this is the only α2(VI) allele expressed in significant quantities in UCMD21 fibroblasts, we used these fibroblasts to study the effect of the α2(VI) C1 domain deletion on collagen VI biosynthesis and identified a critical role for the α2(VI) C1 domain in collagen VI microfibril assembly.

**EXPERIMENTAL PROCEDURES**

**Muscle Biopsy Staining**—Frozen sections (8 μm), cut from muscle biopsies using a CM1900 Cryostat (Leica), were mounted onto Superfrost® Plus slides (Menzel-Glaser) and immunostained with collagen VI and perlecan antibodies as described previously (24). The images were obtained using an Olympus BX50F4 microscope at 40× magnification.

**RNA and DNA Extraction, PCR and Sequencing**—Total RNA was extracted from control and patient fibroblasts using RNeasy (Qiagen). RNA was reverse transcribed using murine leukemia virus reverse transcriptase and an oligo(dT) primer according to the manufacturer’s protocol (GeneAmp RNA PCR kit; Applied Biosystems). The resulting cDNA was used as a template for PCR amplification and sequencing of the coding regions of α1(VI), α2(VI), and α3(VI), as described previously (17). Genomic DNA was extracted from patient fibroblasts and from blood samples obtained from both parents. COL6A2 exons 24 and 25, containing the mutations, were PCR-amplified and sequenced using a forward primer in intron 23, 5′-TCAGAGAGCAAGATCAGTGG-3′, and a reverse primer in intron 25, 5′-GGACTTCTCCCATCTCATGC-3′. COL6A2 exon 26 was PCR-amplified and sequenced using the forward primer 5′-ATGAGTGGGAGAACGCAG-3′ and reverse primer 5′-ACATTATCCCTCAGGTTGAG-3′. The collagen VI α1(VI), α2(VI), and α3(VI) mRNAs were analyzed by Northern blot as described previously (17). A 270-bp region of α2(VI) mRNA (c.16600–1930) harboring the mutations identified in the patient was amplified by RT-PCR using the primers 5′-AAAGGAGAGCCCTGCAGATCC-3′ and 5′-TGGTACACAGTTGATGACG-3′ and analyzed by agarose gel electrophoresis.

**Collagen VI Biosynthetic Labeling and Immunoprecipitation**—Patient and control fibroblasts were grown to confluence in 10-cm² dishes and incubated overnight in DMEM with 10% FBS containing 0.25 mM sodium ascorbate. Fibroblasts were radiolabeled for 18 h with 100 μCi/ml [35S]methionine (Tran35S-label 1032 Ci/mmol; ICN Pharmaceuticals, Inc.) in 750 μl of serum-free and methionine-free DMEM containing 0.25 mM sodium ascorbate. The cell and medium fractions were harvested, and collagen VI was immunoprecipitated with an α3(VI) N1 domain antibody and analyzed under reducing and nonreducing conditions as described previously (18). For pulse-chase analysis, the fibroblasts were radiolabeled, as above, for 30 min, and chased for 0, 0.5, 1, 2, 4, or 8 h in serum-free DMEM containing 0.25 mM ascorbate and 10 μM L-methionine. Collagen VI was immunoprecipitated from cell and medium samples with an α2(VI) N1 domain antibody and analyzed under reducing and nonreducing conditions as described previously (18).

**Immunostaining**—Fibroblasts were grown to confluence in four-well chamber slides and supplemented daily with 0.25 mM sodium ascorbate for 2 days. The extracellular matrix was stained for collagen VI, and the cell nuclei were stained with DAPI using methods described previously (18).

**Blue Native-PAGE Analysis of Collagen VI Microfibril Assembly**—Patient and control fibroblasts were grown to confluence in 10-cm² dishes and supplemented overnight with 0.25 mM sodium ascorbate. The cells were then radiolabeled for 18 h with [35S]methionine, as described above, and collagen VI was immunoprecipitated from the medium (11, 18). Collagen VI was eluted into 15 μl of 1× Native-PAGE sample buffer (Invitrogen) and 5 μl of Native-PAGE 5% G-250 sample additive (Invitrogen) at 65 °C for 10 min. Collagen VI microfibrils were analyzed on Native-PAGE 3–12% Novex Bis-Tris gels (Invitrogen) and visualized by autoradiography.

**Size Exclusion Chromatography and Analysis of Collagen VI Microfibril Assembly**—Patient and control fibroblasts were grown to confluence in 10-cm² dishes and incubated overnight in serum-free DMEM containing 0.25 mM sodium ascorbate. Medium was then removed to 1.5-ml tubes, and protease inhibitors were added to the following concentrations: 1 mM AEBSF and 20 mM N-ethylmaleimide, along with a final concentration of 1% Nonidet P-40. A Sephacryl S1000 SF column (47-ml column volume) was equilibrated with running buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA) at 0.5 ml/min. Medium (0.5 ml) was loaded onto the column, and protein was eluted in a total of 75 ml (0.5-ml fractions). To determine which fractions
contained collagen VI, 100 µl of each fraction was dotted onto HyBond LFP (GE Healthcare) using a Millipore dot blotting apparatus. Once dried, the membrane was rinsed twice with 200 µl of PBS and blocked with 5% BSA in PBS at room temperature for 1 h. The membranes were then incubated with a collagen VI polyclonal antibody (Fitzgerald Industries International) at a 1:10,000 dilution in PBS containing 0.5% BSA and 0.01% Tween 20, for 1 h at room temperature. The membranes were washed twice, 5 min each time, with PBS containing 0.1% Tween 20, followed by incubation with Alexa Fluor® 488 goat anti-rabbit secondary antibody (Invitrogen) diluted at 1:5000 in PBS containing 0.5% BSA and 0.01% Tween 20, for 1 h at room temperature. The membranes were then washed four times, 5 min each time, in PBS containing 0.1% Tween 20, followed by a 5-min wash in PBS alone. The membranes were then dried at 37 °C for 30 min, and the fluorescent signal was detected and quantitated using a Typhoon 9400 variable mode imager and ImageQuant software.

**Electron Microscopy**—Confluent human fibroblasts in 75-cm² flasks were incubated for 18 h in serum-free DMEM containing 0.25 mM sodium ascorbate. The media was collected and clarified by centrifugation. The medium (5 µl) was absorbed for 30 s onto carbon-coated Cu400 grids that had been glow discharged for 30 s at 25 mA. The grids were then briefly washed three times with water and negatively stained for 15 s with 2% w/v uranyl acetate. The samples were observed in a Tecnai12 Twin TEM at 80 keV, and the images were recorded on a 2048 × 2048 CCD camera.

**Constructing Expression Vectors Containing the α2(VI) Wild Type and p.V619_I620del2 Mutant C1 Domains**—Wild type and mutant α2(VI) C1 domains (nucleotides 1822–2436) were amplified from control and UCMD21 cDNA using a forward primer containing a 5’ SmaI restriction enzyme site followed by the StrepII tag sequence (25), and a reverse primer containing a 3’ HindIII restriction enzyme site. PCR products were purified and sequenced, and digestionally digested with SmaI and HindIII (New England Biolabs). Digested products were purified from a 1% (w/v) agarose gel and ligated into the episomal expression vector pCEP4-BM40-HisEK that had also been digested with PvuII (compatible with SmaI) and HindIII. This vector has previously been modified from the pCPE4 vector (Invitrogen) to contain a cleavable BM40 secretory signal peptide at the N terminus of the multiple cloning site (26). Selected clones were sequenced with a pCPE4-specific primer (Invitrogen) to ensure that no errors had been introduced during PCR.

**Expression of Recombinant α2(VI) C1 Domains**—Human embryonic kidney EBNA-293 cells were grown in 10-cm² wells in DMEM with 10% FBS until 90% confluent. The cells were transfected with 2 µg of the wild type or mutant α2(VI) C1 domain expression construct using FuGENE HD (Roche Applied Sciences) at a ratio of 7:2 (FuGENE HD (µl):DNA(µg)). The cells were incubated with FuGENE-DNA complex for 24 h and were then transferred to an 80-cm² flask for selection in DMEM with 10% FBS containing 250 µg/ml hygromycin B (Roche Applied Sciences). The cells were passaged twice to ensure complete selection, and then 2 × 10⁶ cells were plated in 10-cm² wells containing 2 ml of DMEM with 10% FBS containing 250 µg/ml hygromycin B. After 24 h of incubation, 1 ml of medium was removed to 1.5-ml tubes, and protease inhibitors were added to the final concentrations: 20 mM N-ethylmaleimide and 1 mM AEBSF. The medium was cleared of cellular debris by centrifugation at 13,000 × g for 30 min at 4 °C. The cell layer was rinsed twice in PBS, collected in 500 µl of lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1 mM AEBSF, 20 mM N-ethylmaleimide, 1% Nonidet P-40), and fractionated by centrifugation at 13,000 × g for 30 min at 4 °C. The soluble cell lysate was retained, and the insoluble pellet was resuspended in 100 µl of urea/thiourea buffer (7 M urea, 2 M thiourea, 30 mM Tris, pH 8.5, 4% CHAPS).

**Western Blotting of Recombinant α2(VI) C1 Domains**—Aliquots of the insoluble cell fraction, the soluble cell fraction, and the medium harvested from transfected EBNA-293 cells and an untransfected control were incubated at 65 °C for 10 min in a final concentration of 1× NuPAGE LDS sample buffer (Invitrogen). The samples were analyzed on NuPAGE Novex 4–12% Bis-Tris precast acrylamide gels (Invitrogen) alongside a Precision Plus protein standard (Bio-Rad). The proteins were transferred to nitrocellulose membranes (GE Healthcare) overnight at 100 mA at 4 °C. The membrane was blocked in 5% BSA (w/v) in PBS for 1 h at room temperature and then incubated in PBS containing 0.5% skim milk powder, a 1:1000 dilution of Strept tag antibody (Qiagen), and 0.01% Tween 20 (Sigma) for 1 h at room temperature. The membrane was washed four times, 10 min each, in PBS containing 0.1% Tween 20 and then incubated at room temperature for 1 h in PBS containing 0.5% skim milk powder, a 1:10,000 dilution of horseradish peroxidase-conjugated anti-mouse antibody (Dako), and 0.01% Tween 20. This was followed by six 5-min washes in PBS containing 0.1% Tween 20. The membrane was then exposed to Amersham Biosciences Plus ECL Western blotting detection reagents (GE Healthcare) for 5 min, and proteins were visualized by exposure to Amersham Biosciences high performance chemiluminescent film (GE Healthcare).

**RESULTS**

**Clinical Details**—The patient, UCMD21, presented at birth with hypotonia and bilateral hip dislocation. She has torticollis; marked wrist and finger hyperlaxity, elbow, knee, and finger contractures; proximal muscle weakness; and weak facial, pelvic girdle, and neck flexor muscles. When reviewed at 18 months she was not walking and had reached the motor milestones expected for a 6-month-old child. Her clinical presentation was consistent with a diagnosis of Ullrich congenital muscular dystrophy. Her parents are clinically unaffected and are not related. To confirm the diagnosis of a collagen VI disorder, control and patient muscle biopsies were stained with perlecan and collagen VI antibodies (Fig. 1). Perlecan localizes to the basement membrane surrounding muscle fibers. Collagen VI largely co-localizes with perlecan in the control muscle biopsy; however, in UCMD21 collagen VI does not co-localize with perlecan but is predominantly in the interstitial matrix surrounding muscle fibers (Fig. 1). This abnormal localization of collagen VI is a characteristic finding in muscular dystrophy patients with collagen VI mutations (18, 27, 28).

**UCMD21 Has Recessive COL6A2 Mutations**—The α1(VI), α2(VI), and α3(VI) mRNAs were amplified by RT-PCR and sequenced. This revealed compound heterozygous α2(VI)
An α2(VI) VWA Mutation Prevents Microfibril Formation

FIGURE 1. Muscle biopsy immunostaining for collagen VI. Frozen sections of control and patient (UCMD21) muscle were stained with antibodies to collagen VI (red) and perlecan (green). In the control muscle biopsy, collagen VI and perlecan co-localize in the basement membrane surrounding muscle fibers (yellow). In UCMD21, collagen VI no longer co-localizes with perlecan but is seen predominantly in the interstitial space between muscle fibers.

mutations. The patient inherited a c.1771−1G→T substitution in intron 23 from her unaffected father (Fig. 2A). This leads to skipping of exon 24 (r.1771_1816 del46; data not shown), and a shift in the open reading frame from Thr590 that introduces a premature termination codon 148 amino acids downstream (Fig. 2B). On the alternate allele, inherited from her unaffected mother, is a c.1855_1860del6 mutation in exon 25 that leads to an in-frame two-amino acid deletion in the C1 VWA domain (p.V619_I620del2; Fig. 2A).

To determine whether the mRNA containing the exon 24 skip and a premature stop codon was degraded by nonsense-mediated mRNA decay, we examined the relative amounts of the α1(VI), α2(VI), and α3(VI) mRNAs by Northern blot. In control fibroblasts, α1(VI) and α2(VI) mRNAs are expressed at similar levels (Fig. 3A); however, in UCMD21 fibroblasts the relative amount of α2(VI) mRNA is reduced in comparison with α1(VI), indicating that some α2(VI) mRNA is degraded. To determine which allele is subject to mRNA degradation, we amplified a 271-bp region of α2(VI) mRNA containing both mutations by RT-PCR (Fig. 3B). In the patient, the amplification product harboring the six-base pair deletion (265-bp product) is indistinguishable from the 271-bp wild type product seen in the control. Amplification of the mRNA harboring the exon 24 deletion yielded a 225-bp product; however, the intensity of this band was significantly reduced in comparison with the 265-bp product. This confirms that mRNA containing the exon 24 skip is subject to mRNA degradation. Thus, the allele harboring the V619_J620 deletion is the predominant α2(VI) allele expressed in patient fibroblasts.

We also identified other sequence variations in the UCMD21 RT-PCR products (Table 1). All but one of these changes have been identified previously in unaffected individuals, and we considered that they were likely nonpathogenic. One heterozygous sequence variation carried by UCMD21, COL6A2 c.2351G→A (p.R784H), was found in a UCMD patient who also carried a pathogenic heterozygous COL6A1 p.G290R mutation (29) but has not been reported in unaffected individuals. UCMD21 inherited the COL6A2 c.2351G→A variation from her father, who is a heterozygous carrier of the change (data not shown). This means that the variation is on the same COL6A2 allele as the c.1771−1G→T mutation that causes exon 24 skipping. Because the variation is downstream of the frameshift, it will not be expressed at the protein level and will not contribute to the phenotype in UCMD21.

UCMD21 Fibroblasts Assemble and Secrete Tetramers—To determine the biosynthetic consequences of the α2(VI) C1 domain deletion, we radiolabeled control and UCMD21 fibroblasts overnight with [35S]methionine, immunoprecipitated collagen VI from the cell and medium fractions with an α3(VI) N1 domain antibody, and analyzed it by gel electrophoresis under reducing and nonreducing conditions. Reduced collagen VI was resolved on 3–8% polyacrylamide gradient gels, revealing that α1(VI) and α2(VI) chains co-immunoprecipitate with α3(VI) chains in the cell and medium of patient fibroblasts.

FIGURE 2. UCMD21 has heterozygous recessive α2(VI) mutations. A, genomic DNA PCR and sequencing. The patient, UCMD21, has compound heterozygous mutations. The first, inherited from her unaffected father, is a c.1771−1G→T splice site mutation that leads to skipping of exon 24 during pre-mRNA splicing (data not shown). The second change, inherited from her unaffected mother, is a c.1855_1860del6 mutation causing an in-frame p.V619_I620del2 mutation in the N-terminal region of the α2(VI) C1 VWA domain. B, schematic representation of the α2(VI) protein changes. The N1, C1, and C2 VWA domains are labeled, and the triple helical domain is shown as a black line. Exon 24 skipping causes a reading frameshift, starting from p.T590 at the C-terminal end of the triple helix, and introduces a premature stop codon 148 amino acids downstream. The abnormal protein sequence at the C-terminal end of the α2(VI) chain is indicated in red. The p.V619_I620del2 mutation is near the N-terminal end of the C1 VWA domain.
Collagen VI intracellular assembly and secretion in UCMD21 were examined. The effect of the α2(VI) and α3(VI) N1 domain antibody that recognizes unassembled α2(VI) chains as well as α2(VI) chains that have been incorporated into monomers, dimers, and tetramers. When collagen VI was analyzed under reducing conditions on 3–8% Tris-acetate gels, significant amounts of α3(VI) are first seen to co-immunoprecipitate with α2(VI) chains at 0.5 h in the cell of control fibroblasts (Fig. 5A). However, in the cell of UCMD21 fibroblasts, significant amounts of α3(VI) chains only co-immunoprecipitate with α2(VI) at 1 h (Fig. 5A), indicating that the C1 domain deletion delays the association of mutant α2(VI) chains with α1(VI) and α3(VI). Furthermore, the amount of co-immunoprecipitated α3(VI) in control cells is greatest at 1 h (Fig. 5A), after which the amount of α3(VI) chains in the cell progressively declines as assembled collagen VI is secreted into the medium (Fig. 5B). By contrast, the amount of co-immunoprecipitated α3(VI) in the cell of UCMD21 fibroblasts remains at its greatest from 2 to 4 h, and assembled collagen VI does not appear in the medium until 2 h (Fig. 5B), indicating that the deletion affects the rate at which tetramers are secreted. To determine whether the deletion also affects the rate at which dimers and tetramers are assembled, collagen VI was analyzed under nonreducing conditions. This revealed that dimers first appear in the cell of control fibroblasts at 0.5 h, and tetramers at 1 h (Fig. 5C). Tetramers are in the medium of control fibroblasts at 1 h, and the amount in the medium does not increase dramatically after 2 h, indicating that most of the radiolabeled protein is secreted by this time point (Fig. 5E). By contrast, in UCMD21 fibroblasts, dimers first appear in the cell at 1 h, and tetramers first appear at 2 h (Fig. 5D). Significant secretion of tetramers is not apparent until 2 h (Fig. 5F), and the amount of tetramers in the medium is greatest at 8 h. Collectively, these data confirm that

### FIGURE 4. Collagen VI biosynthetic analysis in UCMD21 fibroblasts. Control (lane C) and UCMD21 (lane U21) fibroblasts were labeled overnight with [35S]methionine, and collagen VI was immunoprecipitated from the cell and medium with an α3(VI) N1 domain antibody. A, immunoprecipitated collagen VI was analyzed under reducing conditions on 3–8% Tris-acetate gels to resolve the constituent α1(VI), α2(VI), and α3(VI) chains. B, tetramers are the predominant form of collagen VI secreted into the medium. Nonreduced immunoprecipitated collage VI samples were analyzed on 4% acrylamide, 0.5% agarose gels to visualize collagen VI monomers, dimers, and tetramers. Collagen VI dimers and tetramers and unassembled α3(VI) chains are indicated as fibronectin dimers (Fn2). UCMD21 mutant α2(VI) chains are incorporated into stable tetramers that are secreted from the cell. Some unassembled α3(VI) chains are present in the UCMD21 cell fraction, suggesting that they are present in excess. This is consistent with data demonstrating that fewer α2(VI) chains are synthesized because the mRNA from one allele is degraded.

### FIGURE 3. mRNA containing the exon 24 skip and a premature stop codon is degraded by nonsense-mediated mRNA decay. A, Northern blot analysis. Total RNA from control (lane C) and UCMD21 (lane U21) fibroblasts was separated on a denaturing agarose gel, transferred to nitrocellulose, and probed with [α-32P]dCTP-labeled α1(VI), α2(VI), and α3(VI) cDNA probes. In the control, α1(VI) and α2(VI) mRNAs are present in similar amounts; however, in UCMD21, α2(VI) mRNA is reduced relative to α1(VI) mRNA, indicating mRNA decay. B, RT-PCR analysis of a 271-bp fragment of α2(VI) that includes both mutations. In UCMD21 fibroblasts, amplification of the α2(VI) mRNA carrying the V619_I620 deletion generates a 265-bp product that is indistinguishable from the 271-bp wild type product in control fibroblasts. Amplification of the mRNA carrying the exon 24 skip generates a faint 225-bp product in UCMD21. The reduced amount of this RT-PCR product indicates that the mRNA, which contains a premature stop codon, is subject to mRNA degradation. The band migrating above the 265-bp product in the UCMD21 sample is a heteroduplex (het) of the 265- and 225-bp products.

### TABLE 1 Collagen VI sequence variations in UCMD21

| Nucleotide change | Exon, Domain | Amino acid change | Status in U21 | Reference |
|-------------------|-------------|------------------|---------------|-----------|
| COL6A1            | 15, helix c.1095 C → T | p.G365G | hetero | 29 |
|                  | 15, helix c.1095 C → T | p.G365G | hetero | 29 |
|                  | 35, C2     | c.2549 G → A    | p.R850H | hetero | 29 |
|                  | 35, C2     | c.2667 G → A    | p.A889A | hetero | 29 |
|                  | 35, C2     | c.2797C → T    | p.S932S | hetero | 29 |
|                  | 35, C2     | c.2797C → T    | p.S932S | hetero | 29 |
|                  | 24, linker | c.1771, 1816del46 | p.T590fs | hetero | This Study |
|                  | 24, linker | c.1771, 1816del46 | p.T590fs | hetero | This Study |
|                  | 26, C1     | c.2351G → A    | p.R784H | hetero | 29 |
|                  | 26, C1     | c.2351G → A    | p.R784H | hetero | 29 |
|                  | 28, C2     | c.2697T → G    | p.T899T | hetero | 29 |
|                  | 28, C2     | c.2697T → G    | p.T899T | hetero | 29 |
| COL6A2            | 3, N1     | c.679 A → G    | p.N227D | homo | 18 |
|                  | 24, linker | c.1771, 1816del46 | p.T590fs | hetero | This Study |
|                  | 25, C1     | c.1855, 1860del6 | p.V619, I620del2 | hetero | This Study |
|                  | 25, C1     | c.1855, 1860del6 | p.V619, I620del2 | hetero | This Study |
|                  | 26, C1     | c.2351G → A    | p.R784H | hetero | 29 |
|                  | 26, C1     | c.2351G → A    | p.R784H | hetero | 29 |
|                  | 28, C2     | c.2697T → G    | p.T899T | hetero | 29 |
|                  | 28, C2     | c.2697T → G    | p.T899T | hetero | 29 |
| COL6A3            | 20, helix | c.6369 G → A    | p.L2123L | hetero | 29 |
|                  | 28, helix | c.6855 G → C    | p.G2285G | homo | 29 |
|                  | 30, helix | c.6945 T → C    | p.F2315F | homo | 29 |
|                  | 36, C1     | c.7292 T → A    | p.V2431D | homo | 45 |
|                  | 38, C2     | c.7842 T → C    | p.S2614S | homo | 29 |
|                  | 38, C2     | c.8451 G → A    | p.P2817P | homo | 45 |
|                  | 40, C3     | c.8780 C → T    | p.T2927M | homo | 45 |
|                  | 40, C3     | c.8820 A → G    | p.T2940T | homo | 43 |
|                  | 40, C3     | c.8874, 8875insGCT | p.A2958_A2990insA | homo | 43 |
|                  | 40, C3     | c.8959 G → A    | p.V2987M | homo | 45 |

* Sequence variations resulting in single amino acid substitutions are indicated in bold. Pathogenic mutations are italicized.
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**FIGURE 5.** Pulse-chase analysis of collagen VI assembly and secretion. Control and UCMD21 fibroblasts were labeled with \[^{35}\text{S}\]methionine for 30 min and chased for up to 8 h. Collagen VI was immunoprecipitated with an \( \alpha2(\text{VI}) \) N1 domain antibody. \( A \), collagen VI immunoprecipitated from the cell layer was analyzed under reducing conditions on a 3–8% gradient acrylamide gel. In the control, \( \alpha3(\text{VI}) \) chains were co-immunoprecipitated from 0.5 h, indicating that they had assembled with \( \alpha2(\text{VI}) \) chains. In UCMD21, assembly of the mutant \( \alpha2(\text{VI}) \) chains with \( \alpha3(\text{VI}) \) was delayed with significant co-immunoprecipitation occurring only after 1 h of chase. \( B \), collagen VI immunoprecipitated from the medium was resolved under reducing conditions on a 3–8% gradient acrylamide gel. The secretion of collagen VI from control cells can be seen after 1 h of chase, reaching a maximum between 2 and 4 h of chase. In contrast, secretion was much slower from UCMD21 cells, with most of the protein appearing in the medium between 4 and 8 h of chase. \( C \)–\( F \), in a separate pulse-chase experiment, collagen VI immunoprecipitated from the cell and medium of control and UCMD21 fibroblasts and was analyzed on nonreducing composite 2.4% polyacrylamide, 0.5% agarose gels. \( C \), nonreduced collagen VI immunoprecipitated from the cell of control fibroblasts. Dimers first appear in the cell at 0.5 h, and tetramers first appear at 1 h. \( D \), nonreduced collagen VI immunoprecipitated from the cell layer of UCMD21 fibroblasts. Dimers appear in the cell from 1 h of chase, and tetramers appear from 2 h onward. \( E \), nonreduced collagen VI immunoprecipitated from the medium of control fibroblasts. Tetramers first appear in the medium of control fibroblasts from 1 h, and the majority of radiolabeled protein is present in the medium from 2 h onward. \( F \), nonreduced, collagen VI immunoprecipitated from the medium of UCMD21 fibroblasts. Tetramers appear in the medium after 2 h of chase, and the majority of radiolabeled protein is present in the medium from 8 h. Collectively these data indicate that the \( \alpha2(\text{VI}) \) C1 domain deletion in UCMD21 delays the assembly of collagen VI in fibroblasts, \( F_n_2 \), fibronectin dimer.

in the UCMD21 fibroblasts, the association of mutant \( \alpha2(\text{VI}) \) chains with \( \alpha1(\text{VI}) \) and \( \alpha3(\text{VI}) \) chains is delayed in comparison with controls. However, there is no accumulation of monomers or dimers in the UCMD21 cells, indicating that subsequent assembly of mutant monomers into dimers and tetramers is not significantly altered.

**FIGURE 6.** Immunostaining of the collagen VI matrix deposited by fibroblasts. Control and UCMD21 fibroblasts were grown for 2 days post-confluence in the presence of sodium ascorbate and stained with a collagen VI antibody. The cell nuclei were stained with DAPI. Control fibroblasts deposited an extensive collagen VI network; however, collagen VI was not detected in the matrix of UCMD21 fibroblasts.

The \( \alpha2(\text{VI}) \) C1 Domain Deletion p.V619_I620del2 Prevents Association of Secreted Tetramers into Microfibrils—To assess extracellular collagen VI assembly, control and patient fibroblasts were grown for 2 days post-confluence in the presence of sodium ascorbate to promote deposition of a collagenous extracellular matrix. The cultures were stained with a collagen VI antibody and the nuclear stain, DAPI. Control fibroblasts deposited a dense network of collagen VI microfibrils (Fig. 6). By contrast, there was no detectable collagen VI matrix in UCMD21 cultures, suggesting that mutant tetramers secreted into the medium are unable to associate into microfibrils.

We used a number of independent approaches to confirm the deficiency of collagen VI microfibrils in the medium of control and UCMD21 fibroblasts and to directly measure the efficiency of mutant collagen VI tetramer assembly. Our first approach was to fractionate the medium by gel filtration chromatography on a Sephacryl S1000 SF column and detect the eluted collagen VI on dot blots with a specific antibody. In control medium, collagen VI eluted as a broad peak between fractions 70–81 (Fig. 7A). Although we were unable to completely resolve microfibrils containing different numbers of tetramers, the broad elution profile suggested a range of microfibril sizes was present. UCMD21 collagen VI eluted later than the control, in fractions 75–81, with the main peak in fractions 77–80, demonstrating that although there were some extracellular collagen VI assemblies in the patient, they were much smaller than those in the control, suggesting that tetramer assembly into microfibrils was severely compromised.

Our second strategy for determining the size of the collagen VI microfibrils was to resolve radiolabeled immunoprecipitated collagen VI on Blue Native-PAGE gels (Fig. 7B). At acid pH, collagen VI microfibrils dissociate into single tetramers (30). So, to determine where single tetramers migrated on these gels, we eluted an aliquot of immunoprecipitated protein from the protein A-Sepharose beads with 10 mM glycine, pH 2.5, prior to electrophoresis (data not shown). Single tetramers were relatively rare in the medium of control fibroblasts compared with double tetramers; however, the majority of the collagen VI was present in microfibrils containing three or more noncovalently associated tetramers. In contrast, the collagen VI in UCMD21 medium was predominantly present as single or...
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To further confirm this and more quantitatively assess the microfibril assembly defect, we used electron microscopy to visualize tetramers and microfibrils in the medium of control and UCMD21 fibroblasts and then determined how many tetramers had associated end-to-end in each microfibril. The number of microfibrils containing one to ten or more associated tetramers was expressed as a percentage of the total microfibril population (Fig. 7C). Single tetramers account for 24% of microfibrils in control fibroblasts but almost 70% of the total microfibril population in UCMD21 fibroblasts. In control fibroblasts, microfibrils containing 10 or more tetramers were present, whereas microfibrils with more than three tetramers were not seen in UCMD21. Together, these data indicate that collagen VI tetramers containing α2(VI) chains with the p.V619_I620del2 mutation in the C1 domain have severely compromised microfibril assembly. The Recombinant α2(VI) p.V619_I620del2 C1 Domain Is Misfolded—To assess the effect of the α2(VI) C1 VWA domain mutation on domain structure and folding, StreptII-tagged wild type and mutant α2(VI) C1 domains were expressed in EBNA-293 cells. The medium and the insoluble and soluble cell fractions were harvested from transfected cells and analyzed under nonreducing conditions by Western blot using a primary antibody to the StreptII tag (Fig. 8). The StreptII antibody did not recognize protein from untransfected EBNA-293 cells (data not shown), confirming specificity for the StreptII tag. The recombinant wild type α2(VI) C1 domain was soluble and was predominantly detected as a monomer in the medium of transfected cells as has been shown previously for correctly folded VWA domains expressed in mammalian cells (31). By contrast, the mutant α2(VI) C1 domain was not secreted into the medium and was predominantly found in the insoluble cell
fraction where it formed aggregates with molecular weights consistent with two, three, four, five, and more disulfide-bonded C1 domains, indicating that the α2(VI) C1 domain deletion has detrimental effects on the folding and overall structure of the C1 domain. There was a small amount of protein of ~250 kDa in all three fractions of both the wild type and mutant cell lines. We predict that these high molecular weight proteins are misfolded C1 domains that are forming intermolecular disulfide bonds, a plausible explanation given the high level of recombinant expression. These data provide evidence that the p.V619_I620del2 mutation disrupts the folding and the structure of the C1 domain.

**DISCUSSION**

Analysis of collagen VI biosynthesis in fibroblasts of patients with naturally occurring mutations in the collagen VI genes provides a useful model for defining regions that are involved in collagen VI assembly. We have presented a UCMD patient, UCMD21, who has compound heterozygous mutations in COL6A2. The first mutation is a single nucleotide substitution at the −1 position of intron 23, which leads to exon 24 skipping and changes the open reading frame. This frameshift is predicted to introduce a premature termination codon 148 amino acid residues downstream. Only residual amounts of mRNA from this allele are amplified by RT-PCR, indicating that the transcript is subject to nonsense-mediated mRNA decay. Skipping from this allele are amplified by RT-PCR, indicating that the only acid residues downstream. Only residual amounts of mRNA that escape degradation lead to expression of an α2(VI) chain that has an intact triple helical sequence with a missense C-terminal region of 148 amino acids. Despite the absence of a normal C-terminal region, mutant α2(VI) chains were reported to associate into triple helical monomers, but no evidence was presented suggesting that mutant monomers further assembled into dimers and tetramers (34). In our experiments we did not detect a smaller α2(VI) protein consistent with the exon 24 skip, indicating that the only α2(VI) chains made in significant amounts are products of the other COL6A2 allele.

The second mutation in UCMD21 is a six-base deletion resulting in expression of an α2(VI) chain with a two-amino acid deletion in the N-terminal region of the C1 domain, p.V619_I620del2. We have shown that the mutant α2(VI) chains are stable and are incorporated into triple helical monomers, dimers, and tetramers that are secreted. It is outside of the cell, however, that mutant tetramers display a severe reduction in their ability to form microfibrils. To investigate the consequences of the two-amino acid deletion on the folding and structure of the α2(VI) C1 domain, we expressed wild type and mutant domains in mammalian cells. The same approach was previously used to assess the effect of a Bethlem myopathy α3(VI) N2 domain G1679E mutation on domain stability and structure (27). Recombinant wild type α3(VI) N2 domains are autonomously folded units efficiently secreted into the medium of transfected cells, but the G1679E substitution causes misfolding of the domain, resulting in intracellular retention and degradation (35). We have shown that recombinant wild type α2(VI) C1 domains are also predominantly present in the medium of transfected cells. However, α2(VI) C1 domains carrying the p.V619_I620del2 mutation are retained within the cell where they form aberrant insoluble disulfide-bonded aggregates. A similar result was reported for recombinant matrilin-3 VWA domains with multiple epiphyseal dysplasia amino acid substitutions (31). Wild type matrilin-3 VWA domains are present in the medium of transfected cells, as are VWA domains carrying a nonsynonymous polymorphism. By contrast, VWA domains expressing disease-causing missense mutations existed primarily in an unfolded state and were retained intracellularly where they formed disulfide-bonded multimers (31). The misfolded matrilin-3 VWA domains, when expressed within the recombinant full-length protein, lead to intracellular retention and aggregation of the mutant full-length protein (31). In contrast, collagen VI biosynthetic analysis in UCMD21 fibroblasts has revealed that the mutant α2(VI) chain maintains the ability to associate with α1(VI) and α3(VI) chains to form stable triple helical structures, despite containing a misfolded C1 domain.

Chain selection and association is the initial step in the assembly of all collagens, and there is much evidence to suggest that the C-terminal region of the constituent α-chains is critical for this process (see Ref. 36 for review). In the case of collagen VI, chains truncated after the C1 domain retain the ability to form monomers (10, 11, 23), suggesting that the C1 domains are the site at which the three collagen VI α-chains come together to initiate monomer formation; however, a minor role for the C2 domain in monomer assembly cannot be excluded because the efficiency of monomer formation by chains lacking C2 has not been examined (10, 11, 23). Misfolding of the α2(VI) C1 domain in UCMD21 does not prevent mutant chains associating with α1(VI) and α3(VI) to form triple helical monomers; however, it does slow the rate of monomer formation, as seen by the delay in co-immunoprecipitation of the three chains in pulse-chase experiments. These data suggest that regions other than the collagen VI C1 domains may be important in chain selection and association. Consistent with this, an engineered glycine substitution in the C-terminal region of the α3(VI) triple helix severely impairs the ability of mutant α3(VI) chains to associate with α1(VI) and α2(VI) (20). Collectively, these data suggest that chain association and monomer formation is a cooperative process that involves multiple regions of the collagen VI chains, including the C1 domain and the C-terminal region of the triple helix.

Monomer formation in collagen VI is followed by the intracellular assembly of dimers and tetramers. Tetramers are then secreted from the cell where they align end-to-end to form microfibrils. The assembly of dimers, tetramers, and microfibrils also involves multiple regions of the molecule (see Ref. 16 for review). During microfibril assembly, it is predicted that the end-to-end association of tetramers relies on interactions between the N-terminal triple helical regions of one tetramer.
and the outer globular domains of the adjacent tetramer (5, 9). This idea is supported by reduced microfibril assembly in Beth-
lem myopathy and UCMD patients with glycine substitutions in the N-terminal region of the triple helix, and in cells express-
ing recombinant α3(VI) chains with an engineered N-terminal triple helical glycine substitution (20). The precise interactions that occur between the triple helical region of one tetramer, and the globular domains of another are yet to be fully defined; however, expression of recombinant α3(VI) chains with sequential N-terminal VWA domain deletions showed that the N5 domain is critical for microfibril formation (37). Efficient microfibril formation is also dependent on the α3(VI) C5 domain (11). This study demonstrates for the first time the importance of a correctly folded α2(VI) C1 domain in microfibril assembly. The C1 domain may be directly involved in inter-
actions that facilitate microfibril assembly, or the misfolded domain may impede assembly by altering the spatial arrange-
ment of other interacting domains.

The pathogenic consequences of collagen VI VWA domain mutations are varied. In this study we have shown that a two-
amino acid deletion in the α2(VI) C1 domain has detrimental effects on the structure of the domain but does not affect incor-
poration of the whole chain into triple helical monomers, dimers, and tetramers. Secreted tetramers are not able to form microfibrils and pathology results because there are no collagen VI supramolecular structures in the extracellular matrix. Mon-
omer, dimer, and tetramer formation are also not affected by the α2(VI) C1 domain substitution, p.E624K (38). In contrast to UCMD21, secreted tetramers with the α2(VI) p.E624K muta-
tion form microfibrils in the extracellular matrix, although when viewed by electron microscopy they appear disorganized. These data indicate that α2(VI) p.V619_I620del2 and p.E624K mutations are in the same region of the domain, they have different effects on folding, structure, and interac-
tions critical for microfibril assembly. Interestingly, the p.E624K and p.V619_I620del2 mutations lie directly within, or immediately adjacent to, the DXSXS consensus sequence (621DSSES625) that is part of a conserved metal ion-dependent adhesion site (MIDAS). Binding of divalent cations, commonly Mg2+, to MIDAS motifs is thought to increase the specificity of ligand binding. This idea is supported by the finding that mutations in the conserved MIDAS residues of integrin VWA domains decrease ligand affinity (39–42). Given these data, it is possible that the MIDAS motif of the α2(VI) C1 domain may play a role in facilitating protein interactions critical for normal microfibril assembly. The functionality of the α2(VI) C1 domain MIDAS motif, however, remains to be investigated.

Although the p.V619_I620del2 mutation in the C1 domain does not affect protein stability or tetramer formation, a num-
ber of single amino acid changes in the collagen VI VWA domains have a dramatic effect on folding and/or stability of the mutant chains. To date, the mutations in this class are all in the α2(VI) C2 domain and include p.L873P, p.R876S, p.N897del, and p.P932L (17, 18, 38). α2(VI) chains expressing these mutations are not able to assemble into triple helical structures. By contrast, other mutations in the α3(VI) N2 domain, p.G1679E and p.L1726R, have no obvious effect on intracellular or extra-
cellular collagen VI assembly (17, 44) and are likely to cause pathology by compromising protein-protein interactions in the extracellular matrix. It is evident that collagen VI VWA domain mutations have differing pathogenic consequences depending on the domain involved, the position of the mutation within the domain, and the role of the domain in collagen VI assembly.

Our data provide new mechanistic insights into the complexities of collagen VI intracellular and extracellular assembly. We show that α2(VI) chains with misfolded C1 domains are still able to associate with α1(VI) and α3(VI), providing further evidence that chain selection and assembly of collagen VI monomers is not directed solely by the C1 domains but is a co-oper-
ative process involving multiple protein interactions. We demonstrate for the first time the critical importance of a cor-
rectly folded α2(VI) C1 domain for microfibril assembly. Fur-
ther detailed studies are required to precisely map the organi-
zation of the collagen VI VWA domains in tetramers and microfibrils and further understand the important roles they play in assembly and matrix organization. Such studies will increase our understanding of the pathogenic consequences of the collagen VI VWA domain mutations in muscular dystrophies.

Acknowledgment—We thank the Electron Microscopy Facility, Fac-
ulty of Life Sciences, University of Manchester, for technical support.

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