The C5 Domain of the Collagen VI α3(VI) Chain Is Critical for Extracellular Microfibril Formation and Is Present in the Extracellular Matrix of Cultured Cells*

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Collagen VI, a microfibrillar protein found in virtually all connective tissues, is composed of three distinct subunits, α1(VI), α2(VI), and α3(VI), which associate intracellularly to form triple helical heterotrimeric monomers then dimers and tetramers. The secreted tetramers associate end-to-end to form beaded microfibrils. Although the basic steps in assembly and the structure of the tetramers and microfibrils are well defined, details of the interacting protein domains involved in assembly are still poorly understood. To explore the role of the C-terminal globular regions in assembly, α3(VI) cDNA expression constructs with C-terminal truncations were stably transfected into SaOS-2 cells. Control α3(VI) N6-C5 chains with an intact C-terminal globular region (subdomains C1-C5), and truncated α3(VI) N6-C1, N6-C2, N6-C3, and N6-C4 chains, all associated with endogenous α1(VI) and α2(VI) to form collagen VI monomers, dimers and tetramers, which were secreted. These data demonstrate that subdomains C2-C5 are not required for monomer, dimer or tetramer assembly, and suggest that the important chain selection interactions involve the C1 subdomains. In contrast to tetramers containing control α3(VI) N6-C5 chains, tetramers containing truncated α3(VI) chains were unable to associate efficiently end-to-end in the medium and did not form a significant extracellular matrix, demonstrating that the α3(VI) C5 domain plays a crucial role in collagen VI microfibril assembly. The α3(VI) C5 domain is present in the extracellular matrix of SaOS-2 N6-C3 expressing cells and fibroblasts demonstrating that processing of the C-terminal region of the α3(VI) chain is not essential for microfibril formation.

Collagen VI is composed of three genetically distinct subunits, α1(VI), α2(VI), and α3(VI), each of which contain a relatively short triple helix (~335 amino acids), and N- and C-terminal globular regions. The α1(VI) and α2(VI) chains are similar in size and contain one N- and two C-terminal subdomains of around 200 amino acids that show homology to the type A domains of von Willebrand factor (12). The α3(VI) chain is much larger than the other two, containing up to ten N-terminal A subdomains (N10-N1), and two C-terminal A subdomains (C1 and C2) (13). Recombinant expression of an α3(VI) N9-N2 fragment demonstrated that each A domain is an autonomous folding unit (14). In addition, the α3(VI) chain has three C-terminal subdomains that are not found in α1(VI) or α2(VI). C3 is praline-rich and is similar to some salivary proteins, C4 shows homology to fibronectin type III repeats, and C5, the most C-terminal subdomain, has 40–50% identity to members of the Kunitz family of serine protease inhibitors (13), although it does not have enzyme inhibitory activity (15).

The complex process of collagen VI assembly begins with intracellular association of α1(VI), α2(VI), and α3(VI) chains and folding of the triple helix. These heterotrimeric monomers are stabilized by disulfide bonds that are likely to involve the clusters of cysteine residues that flank the triple helical domain of each chain (12, 13). Unlike the other collagens, collagen VI undergoes further intracellular polymerization prior to assembly and is secreted as a tetramer. The hierarchy of assembly and organization of the monomers in the final secreted tetramers have been defined by biosynthetic studies (16) and detailed electron microscopy examination of the structure of purified collagen VI (17, 18). Dimers form intracellularly by antiparallel alignment of the monomers with a 30-nm stagger, involving interactions of the C-terminal globular region of one monomer with the triple helix of the other. Tetramers, the secreted form of collagen VI, then form by lateral association of dimers. Dimers and tetramers are each linked by two disulfide bonds (18, 19). The final stage of collagen VI assembly occurs outside of the cell where tetramers associate end-to-end to form the beaded microfibrils. Although the basic steps in assembly and the structure of the tetramers and microfibrils are well defined, details of the interacting protein domains involved in assembly are still poorly understood. Some insights have resulted from examination of naturally occurring mutations. Detailed biosynthetic studies on a Bethlem myopathy patient with a heterozygous 18-amino acid deletion toward the N-terminal end of the α1(VI) triple helical domain (amino acids 79–96 of the helix) demonstrated that dimer formation is dependent on the native structure of the triple helix in that region (4). Triple helical glycine mutations that are N-terminal to this region allow dimer and tetramer formation but reduce the efficiency of microfibril formation (20), and exon skipping mutations in the N-terminal end of the triple helix interfere with both tetramer and microfibril formation (10). The use of site-directed mutagenesis to produce alterations in the collagen VI chains is another...
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powerful approach that can be used to identify the protein domains important for correct assembly. Using this approach we have previously shown in transfected SaOS-2 cells that the α3(VI) N5 subdomain is critical for microfibril formation (21). In this study, we have further delineated the α3(VI) subdomains involved in intracellular and extracellular assembly by production and expression of α3(VI) cDNAs containing C-terminal truncations.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis of the α3(VI) Chain—Four α3(VI) cDNA expression constructs containing premature stop codons within the C-terminal domain were produced using strand overlap extension PCR (22). To introduce a stop mutation at amino acid 2599 (α3(VI) protein domains N10-C5, bases and amino acids numbered from the start of translation), bases 7000–7803 and 7783–8102 were amplified by PCR in separate reactions using the α3(VI) N6-C5 plasmid (23) as the template. The overlapping primers each contained two nucleotide substitutions that resulted in the F2599X mutation (TTT-TAG). These primary amplification products were gel-purified and 100 ng of each used as the template in a second round of overlapping PCR. The resulting PCR product (bases 7000–8102) was digested with BsrGI and Eco47III and used to replace the corresponding fragment of the α3(VI) N6-C5 plasmid to produce the expression plasmid α3(VI) N6-C1. Similarly, a stop mutation at residue 2864 was introduced by PCR amplification of bases 8302–8598 and 8584–9172 in which the overlapping primers contained the S2864X (TCA-TAG) change. The second round overlapping PCR product (bases 8302–9172) was digested with Bst1107I and Rsrl II and used to replace the corresponding fragment of a 3-kilobase Apal-NotI subclone in pGEM5 (Promega). The Eco47III-NotI fragment of α3(VI) N6-C5 was replaced with the mutant fragment to produce the expression plasmid α3(VI) N6-C2. The α3(VI) N6-C3 construct was produced by PCR amplification of bases 8302–8997 and 8962–9172. The overlapping primers contained a 3(VI) N6-C5 to produce the α3(VI) N6-C3 plasmid. A similar strategy was used to introduce the S2864X change (CAG-TAG). The second round overlapping PCR product was digested with Bst1107I and Rsrl II and the 775 bp product ligated into the Apal-NotI subclone described above. Finally, the 1950 bp Bst1107I-NotI fragment of this subclone was used to replace the corresponding fragment of α3(VI) N6-C5 to produce the α3(VI) N6-C3 expression plasmid. A similar strategy was used to introduce the M3097X (TCA-TAG) change. The second round overlapping PCR product was digested with Bst1107I and Rsrl II and used to replace the corresponding fragment of α3(VI) N6-C5 to produce the α3(VI) N6-C4 expression plasmid. To confirm the sequence changes and ensure that no errors had been introduced during PCR and cloning the regions of the mutant constructs that were generated by PCR were sequenced (AmpliCycle™, PerkinElmer Life Sciences) (data not shown).

Cell Culture and Transfection—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 (23). 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—SaOS-2 cells were grown to confluence in 10 cm² dishes, incubated overnight in the presence of 0.25 mM sodium ascorbate, then biosynthetically labeled for 18 h with 100 μCi/ml [35S]methionine (Trans-2S-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 added to the following final concentrations: 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), 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% Nonidet P-40, 1 mM AEBSF and 20 mM NEM (cell lysis buffer). Cell lysates and medium samples were clarified by centrifugation, and pre cleared for 1 h with 100 μl of 20% protein A-Sepharose (GE Healthcare). Collagen VI was immunoprecipitated overnight at 4 °C using either an α3(VI) polyclonal antibody made to the recombinant N1 domain, monoclonal antibody 5C6 that recognizes the collagen VI triple helix (24, 25), or a polyclonal antibody made to the recombinant C5 domain of the α3(VI) chain (15) and 100 μl of 20% protein A-Sepharose. The protein A-Sepharose beads were washed twice with NET buffer, 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 3–8% gradient NuPAGE® gels (Invitrogen). Collagen VI triple helical monomers, dimers, and tetramers were analyzed on 2.4% (w/v) acrylamide/0.5% (w/v) agarose composite gels under non-reducing conditions as described previously (23). Radioactively labeled proteins were detected by fluorography (26).

Indirect Immunofluorescence—SaOS-2 cells were grown to confluence in 4-well chamber Permanox slides (Nalge Nunc International) and then supplemented daily for 15 days with 0.25 mM sodium ascorbate. To visualize collagen VI that had been deposited into the extracellular matrix, cell layers were washed with cold PBS² then incubated with either the collagen VI monoclonal antibody 3C4 (24), or the α3(VI) C5 domain antibody (15) for 1 h at 4 °C. Bound antibody was detected using Alexa Fluor® 488 goat anti-rabbit or anti-mouse IgG (Molecular Probes), and the cell layers were then fixed with 4% paraformaldehyde for 15 min. In some experiments cell layers were washed with PBS then fixed with 4% paraformaldehyde for an additional 30 min. The fixed cells were treated with 0.1% Triton X-100 for 20 min then 70% ethanol for 1 min prior to incubation with the primary and secondary antibodies as described above. For co-localization experiments cell layers were incubated simultaneously with 3C4 and C5 and bound antibody detected with Alexa Fluor® 488 goat anti-rabbit and Alexa Fluor® 594 goat anti-mouse IgG. Slides were mounted in FluorSave™ reagent (Calbiochem) and then viewed and imaged using a Zeiss fluorescence microscope or a Leica TCS CP2 S.E. laser scanning confocal microscope.

Human articular cartilage was obtained, from the talus of a 15-year-old boy undergoing surgery for club foot, following informed consent and the approval of the Ethics in Human Research Committee of the Royal Children’s Hospital, Melbourne. The tissue was fixed in Histochoice (Amresco) overnight at 4 °C, then 10-μm sections were digested with 0.2% (w/v) hyaluronidase (bovine, type IV, Sigma) for 15 min to unmask epitopes. Nonspecific binding was blocked with 10% (v/v) normal goat serum and 1% (w/v) bovine serum albumin in PBS for 1 h. Sections were then incubated with 3C4 (1:1000) and C5 (1:500) in PBS with 1% bovine serum albumin for 1 h, washed with PBS and bound antibodies detected with Alexa Fluor® 594 goat anti-rabbit and Alexa

²The abbreviation used is: PBS, phosphate-buffered saline.
RESULTS

Collagen VI Intracellular Assembly in Transfected Cells—To explore the role of the α3(VI) C-terminal globular domains in collagen VI intracellular assembly and extracellular microfibril formation, we produced α3(VI) cDNA expression constructs containing premature stop codons. These constructs were designed to direct the synthesis of α3(VI) chains containing subdomains N6-C1, N6-C2, N6-C3, and N6-C4 (Fig. 1). SaOS-2 cells, which express α1(VI) and α2(VI) mRNA and protein but no α3(VI) mRNA (23), were transfected with the control expression plasmid α3(VI) N6-C5, and the mutant constructs and individual cell clones were selected in medium containing G418.

To determine if the truncated α3(VI) chains were able to assemble intracellularly with α1(VI) and α2(VI), the cell lines were metabolically labeled overnight with [35S]methionine, and collagen VI in the cell and medium was immunoprecipitated. As expected, the α3(VI) chains migrated with a molecular mass consistent with the expected size of the truncated proteins when compared with the control α3(VI) N6-C5 (Fig. 2a), demonstrating that these chains had formed collagen VI heterotrimers that were able to be secreted. Co-assembly of α1(VI) and α2(VI) with α3(VI) N6-C1 chains clearly demonstrated that subdomains C2-C5 may have been specifically cleaved from a proportion of the larger chains by a secreted matrix metalloproteinase (Fig. 2a). The α3(VI) chains migrated with a molecular mass consistent with the expected size of the truncated proteins when compared with the control α3(VI) N6-C5 (Fig. 2a), and the mutant constructs and individual cell clones were selected in medium containing G418.

To determine if the truncated α3(VI) chains were able to assemble intracellularly with α1(VI) and α2(VI), the cell lines were metabolically labeled with [35S]methionine, and collagen VI in the cell and medium was immunoprecipitated with the α3(VI)-specific antibody N1. Samples were analyzed on 3–8% polyacrylamide gels under reducing conditions. Collagen VI tetramers, fibronectin dimers (FN2) in the medium, and intracellular collagen VI monomers are indicated on the right.

In addition to the α3(VI) N6-C5, N6-C4, N6-C3, and N6-C2 chains, we also consistently observed a small amount of immunoprecipitated protein in the medium of these cell lines that had the same migration as α3(VI) N6-C1 (Fig. 2a, lanes 8–12). This band was not immunoprecipitated from the medium of untransfected parental SaOS-2 cells (Fig. 2a, lane 7) indicating that it was an α3(VI) chain that was recognized by the N1 antibody. This suggested that subdomains C2-C5 may have been specifically cleaved from a proportion of the larger chains by a secreted protease. A second band, that co-migrated with the α3(VI) N6-C2, was also consistently seen (Fig. 2a, lanes 9–12) although in much lower amounts than the smaller N6-C1 co-migrating band.

The next stages of collagen VI assembly, the formation of disulfide-bonded dimers and tetramers, were assessed by analysis of the immunoprecipitated proteins on non-reducing agarose/acylamide gels. Tetramers were the predominant form of collagen VI secreted by all of the transfected cell lines (Fig. 2b, lanes 8–12), demonstrating that subdo-
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Microfibril Formation.—To determine which cell lines could incorporate collagen VI into an extracellular matrix, parental and transfected SaOS-2 cells were grown for 15 days post-confluence in the presence of sodium ascorbate. To ensure that only extracellular collagen VI was detected, the cells were stained prior to fixation with the collagen VI antibody 3C4, examined by fluorescence microscopy. An abundant collagen VI matrix was present in cells expressing α3(VI) N6-C5 (Fig. 3f). In contrast, only weak extracellular collagen VI staining was seen in the parental cell line (Fig. 3a), and the cells expressing α3(VI) N6-C1 (Fig. 3b), and α3(VI) N6-C2 (Fig. 3c). This minute amount of collagen VI extracellular matrix deposition is most likely because of the low level of endogenous α3(VI) mRNA expression that is induced after extended culture of these cells in the presence of sodium ascorbate (23). In cells expressing α3(VI) N6-C3 and N6-C4 a small amount of collagen VI extracellular staining was seen (Fig. 3, d and e); however, the staining was much less intense than in the N6-C5-expressing cells. These data demonstrate that while the α3(VI) C3 and C4 domains may play a small role in the end-to-end assembly of tetramers into microfibrils, the C5 domain is critical for efficient microfibril formation.

Secreted collagen VI was also examined by rotary shadowing electron microscopy to visualize the end-to-end assembly of tetramers (Fig. 4A). Microfibril formation in the transfected cell lines was quantified by examination of a large number of micrograph fields (Fig. 4B). The occurrence of “microfibrils” containing 1–10 tetramers is shown as a percentage of the total number of microfibrils. In cells expressing control α3(VI) N6-C5 chains, 86% of the microfibrils contained 2–10 tetramers and only 14% were single tetramers. In stark contrast, almost none of the tetramers containing α3(VI) N6-C2 chains had associated end-to-end with another tetramer and assemblies containing more than three tetramers were never observed. Some association of the N6-C3 and N6-C4 containing tetramers was seen, although at low levels when compared with the N6-C5 containing tetramers. In N6-C3 and N6-C4 cell lines around 70% of the “microfibrils” were single tetramers and around 30% of the microfibrils contained 2–6 tetramers. This is consistent with the small amount of collagen VI immunostaining that was present in the extracellular matrix of these cell lines. Together, the immunostaining and microfibril quantitation data indicate that the α3(VI) C5 subdomain is critical for the interactions between tetramers that promote efficient microfibril formation.

The α3(VI) C5 Domain Is Present in the Extracellular Matrix.—A number of studies have suggested that the C-terminal end of the α3(VI) chain is cleaved after secretion. The most recent have used an antibody specific for the α3(VI) C5 domain and have shown that C5 is present on α3(VI) chains synthesized and secreted by cultured cells, but C5 cannot be detected in the extracellular matrix of cultured cells or tissues (15, 27) suggesting that processing of the α3(VI) chain may be an obligatory step in microfibril formation. The precise cleavage site in the C-terminal domain was not determined in these studies. Our observation that each of the transfected SaOS-2 cell lines contained an α3(VI) band in the medium that had the same migration as the α3(VI) N6-C1 band (Fig. 2a, lanes 8–12) raised the possibility that the protease cleavage site was between the C1 and C2 domains. To explore the nature and origin of the α3(VI) N6-C1 band further, radiolabeled collagen VI secreted by SaOS-2 cells expressing α3(VI) N6-C5 was immunoprecipitated with three different antibodies and analyzed under reducing conditions on polyacrylamide gels. As observed previously, the antibody to the α3(VI) N1 domain immunoprecipitated α3(VI) N6-C5 chains as well as the α3(VI) N6-C1 band (Fig. 5, lane 8), indicating that the N1 domain was

**FIGURE 3.** Collagen VI in the extracellular matrix of SaOS-2 cells. Untransfected SaOS-2 cells (a) and cells transfected with the α3(VI) N6-C1 construct (b), the N6-C2 construct (c), N6-C3 (d), N6-C4 (e), and the control N6-C5 construct (f) were grown for 15 days post-confluence in the presence of 0.25 mM sodium ascorbate. Collagen VI that had been deposited into the extracellular matrix was visualized by incubation with a specific antibody (3C4) prior to fixation. Bound antibody was detected with a fluorescent anti-mouse IgG. Images are ×40.

**FIGURE 4.** Quantitative analysis of collagen VI tetramer-tetramer association. Transfected SaOS-2 cells were incubated for 18 h in serum-free medium containing 0.25 mM sodium ascorbate. The medium was removed, clarified by centrifugation, subjected to mica sandwich squeezing rotary shadowing, and examined by electron microscopy. A, electron micrographs of collagen VI microfibrils in the medium of N6-C5 expressing cells (left) and tetramers in the medium of N6-C1 expressing cells (right). Tetramers are indicated by the arrowheads. The scale bar represents 100 nm. The ability of the tetramers to associate end-to-end was quantitated. The occurrence of microfibrils containing 1–10 tetramers is shown as a percentage of the total number of microfibrils (B). Medium from cells expressing α3(VI) N6-C5 contained collagen VI tetramers that had associated end-to-end to form microfibrils. Medium from cells expressing α3(VI) N6-C2, N6-C3, and N6-C4 contained mainly individual unassociated tetramers, although a small percentage of N6-C3 and N6-C4 tetramers had formed small microfibrils.
mediates as indicated on the that they represent disulfide bonded but non-triple helical collagen VI assembly inter-
fore no evidence that this cleavage is biologically relevant.

of the cleavage sites is between the C1 and C2 domains. There is there-

susceptible to nonspecific proteolytic cleavage in the medium and one

most likely resulted from a cleavage at the N terminus of the chain that

domain, was incorporated into triple helical molecules and therefore

are specifically immunoprecipitated by the N1 and C5 antibodies but not SC6 indicating that they represent disulfide bonded but non-triple helical collagen VI assembly inter-
mediates as indicated on the right. Fibronectin (FN) and fibronectin dimers (FN2) immu-
nnoprecipitated from the medium of untransfected SaOS-2 cells are indicated on the left.

included in this protein. Despite efficient immunoprecipitation of the

α3(VI) N6-C5 chains with the C5 antibody, only minute amounts of the

N6-C1 band could be seen (Fig. 5, lane 9) indicating that this band

lacked the C5 domain and did indeed result from cleavage in the C

terminus of the α3(VI) chain, between C1 and C2. The monoclonal

antibody SC6 recognizes a triple helical collagen VI epitope (24, 25), and

noprecipitated from the medium of untransfected SaOS-2 cells are indicated on the

right. The cleaved α3(VI) N6-C1 band is marked with an asterisk. This band is not precipi-
itated with the triple helical antibody SC6 or the C5 antibody and runs as a single chain

without reduction indicating that it has not been incorporated into triple helical mole-
cules. The reducible bands that migrate above the α3(VI) N6-C5 chains (lanes 11 and 12)

are specifically immunoprecipitated by the N1 and C5 antibodies but not SC6 indicating that they represent disulfide bonded but non-triple helical collagen VI assembly inter-
mediates as indicated on the right. Fibronectin (FN) and fibronectin dimers (FN2) immu-
nnoprecipitated from the medium of untransfected SaOS-2 cells are indicated on the left.

The second minor band that co-migrated with the α3(VI) N6-C5 was

immunoprecipitated with all three antibodies in these experiments (Fig.

5, lanes 7–9). This indicates that this α3(VI) protein retained the C5

domain, was incorporated into triple helical molecules and therefore

most likely resulted from a cleavage at the N terminus of the chain that

removed the N6 domain.

To confirm that the C5 domain was cleaved from the α3(VI) chain in

the SaOS-2 cell culture system, stably transfected SaOS-2 cells express-

ing α3(VI) N6-C5 were grown for 12 days post-confluence in the pres-
ence of sodium ascorbate then stained prior to fixation with the

collagen VI antibodies 3C4 (panels a and c) and C5 (panels b and d). Both antibodies

stained collagen VI in the extracellular matrix of cells expressing α3(VI) N6-C5. Normal

human fibroblasts were grown for 2 days post-confluence and then stained either prior
to fixation (panels e and f) or after fixing with 4% paraformaldehyde (panels g and h) with

3C4 (panels e and g) and C5 (panels f and h). The C5 antibody also stained collagen VI in
the extracellular matrix of fibroblasts indicating that cleavage of the C5 domain is not
required for microfilament formation. Images are ×20. b, SaOS-2 N6-C5 cells (panels a–c)

were stained simultaneously with the 3C4 (panel a) and C5 (panel b) antibodies. An

overlay (panels c) shows co-localization of the epitopes. Images are ×40. The matrix
produced by human fibroblasts (panels d–f) was also co-stained with 3C4 (panel d) and
the C5 (panel e) and a single z section was captured by confocal microscopy. An overlay of
the images shows co-localization of the two epitopes. Images are ×63. The no anti-
histone control staining was negative. c, human articular cartilage was co-stained with the
nuclear stain DAPI (panel a), 3C4 (panel b), and C5 (panel c) and viewed using confocal
microscopy. The images show a group of chondrocytes in the deep zone of the articular
cartilage. An overlay of the images (panel d) indicates that the 3C4 epitope (green) is
most abundant in the pericellular matrix, while the C5 epitope (red) is concentrated
intracellularly and near the cell surface. The antigens overlap in the immediate pericel-
lar matrix (yellow). Control sections in which 3C4 was omitted and C5 was replaced with
non-immune rabbit serum were negative.
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FIGURE 7. Specificity of the C5 antibody. To confirm that the epitopes recognized by the C5 antibody are within the C5 domain SaOS-2 cells expressing α3(VI) N6-C5 (lanes 1–3), α3(VI) N6-C4 chains (lanes 4–6), and untransfected SaOS-2 cells that do not express α3(VI) (lanes 7–9) were metabolically labeled overnight with [35S]methionine, and collagen VI in the medium was immunoprecipitated with either the N1 antibody (lanes 1, 4, and 7) or the C5 antibody (lanes 2, 5, and 8). Lanes 3, 6, and 9 show nonspecific binding of fibronectin to the protein A-Sepharose in the absence of primary antibody. Nonspecific binding of fibronectin to protein A-Sepharose can also been seen in lanes 7 and 8 because SaOS-2 cells do not synthesize the α3(VI) chain. Collagen VI α1(VI), α2(VI), and α3(VI) chains and fibronectin (FN) are indicated on the right. The N1 antibody immunoprecipitates both α3(VI) N6-C5 and N6-C4 chains, whereas the C5 antibody immunoprecipitates α3(VI) N6-C5 chains but does not precipitate collagen VI from N6-C4 expressing cells confirming that it recognizes only the C5 domain.

This led to the conclusion that the C5 domain was removed during microfibril assembly (15). Together these data suggested that collagen VI microfibril assembly in SaOS-2 osteosarcoma cells might be significantly different to assembly in fibroblasts and so it was important to confirm that the C5 domain was not present in the fibroblast extracellular matrix. Normal human fibroblasts were grown until confluence then supplemented for 2 days with sodium ascorbate. The extracellular matrix was stained prior to fixation with either the 3C4 or C5 antibody. In contrast to the previous report (15) the unfixed fibroblast extracellular matrix was stained prior to fixation with either the 3C4 or C5 antibody. In contrast to the previous report (15) the unfixed fibroblast extracellular matrix was strongly stained with the C5 antibody (Fig. 6a, panel f).

We also stained the fibroblast matrix following fixation using conditions similar to those used by Mayer et al. (15) and again the C5 antibody clearly recognized collagen VI in the fibroblast extracellular matrix (Fig. 6a, panel h). Dual immunostaining of the SaOS-2 N6-C5 and human fibroblast matrices with 3C4 and C5 confirmed that the two antibodies co-localized and were staining the same extracellular structures (Fig. 6b).

One possible explanation for these apparently contradictory findings was that we had inadvertently used another antibody and not the C5 antibody. To determine that the antibody was specific for the α3(VI) C5 domain SaOS cells expressing α3(VI) N6-C5 and α3(VI) N6-C4 were biosynthetically labeled and the collagen VI in the medium was immunoprecipitated with either the N1 antibody or the C5 antibody. Both antibodies precipitated collagen VI from the N6-C5-expressing cells (Fig. 7, lanes 1 and 2). The N1 antibody also precipitated collagen VI from the N6-C4 medium (Fig. 7, lane 4). However, the C5 antibody failed to precipitate collagen VI from the N6-C4 expressing cells (Fig. 7, lane 5), clearly demonstrating that it is specific for the C5 domain and that the correct antibody was used in the immunostaining experiments. Further confirmation of the antibody specificity was obtained by dual immunostaining of human articular cartilage with the 3C4 and C5 antibodies (Fig. 6c). The C5 epitope was present intracellularly, however the staining was strongest close to the surface of the cell and in the immediate pericellular matrix. 3C4, which recognizes an α3(VI) N-terminal epitope, stained the pericellular matrix, and there was a zone where the two antibodies co-localized in the immediate pericellular matrix (Fig. 6c, panel d). This finding is similar to that reported by Aigner et al. (27) and again confirms the correct identity of our C5 antibody. Our data thus show that the C5 domain of the collagen VI α3(VI) chain is critical for efficient extracellular microfibril formation and is present in the extracellular matrix of both transfected SaOS-2 cells and normal human fibroblasts, as well as the immediate pericellular matrix of articular cartilage.

DISCUSSION

This study provides fundamental new information about the protein domains involved in intracellular collagen VI assembly and the formation of extracellular microfibrils (Fig. 8). Assembly of α1(VI):α2(VI): α3(VI) heterotrimeric monomers then dimers and tetramers proceeds normally in the absence of α3(VI) C2-C5. There is mounting evidence that the initial chain association event in collagen VI assembly involves interactions between the C-terminal globular domains. Nucleation and folding of the triple helix must then proceed from the C to the N terminus. This is consistent with data showing that a Gly-Val substitution near the C-terminal end of the α3(VI) triple helical domain severely impairs assembly of the three chains into monomers, presumably by preventing nucleation of the triple helix, while N-terminal glycine substitutions do not affect monomer formation or prevent helix folding (20). Our data showing that α3(VI) N6-C1 chains are able to participate in triple helical molecules suggests that the critical chain interaction involve the C1 subdomains. This idea is supported by in vitro transcription/translation experiments that used constructs of the three collagen VI chains and showed that chains with just the C1 subdomain at the C terminus formed triple helical monomers (28).

Disease causing mutations can also provide critical information about the role of protein domains in structure and function. To date, four dominant and four recessive disease causing structural mutations have been identified in the C-terminal globular domains of collagen VI. All of these mutations are in the α2(VI) chain. The dominant Bethlem myopathy mutations within the α2(VI) C1 subdomain include p.D650_H651del (29), a mutation leading to the insertion of sixteen amino acids (p.C605_D606ins16), and a mutation that deletes the first 41 amino acids encoded by exon 26 (p.T656_A698del41) (30, 31). A two base pair insertion in the last exon of COL6A2 leads to a reading frameshift (p.Q889fs) and a premature stop codon (30). Because premature stop codons in the last exon do not normally cause nonsense-mediated decay of the mutant mRNA (32) this mutation is likely to result in production of a truncated α2(VI) chain with an abnormal C2 domain. Recessive mutations, identified in Ullrich congenital muscular dystrophy patients, include a two amino deletion in α2(VI) C1 (p.I759_G760del2) (33), and an exon 27 skipping mutation that deletes 13 amino acids (p.D808_T820del13) in the region that links the C1 and C2 subdomains (30). The effects of these mutations on stability and assembly of the mutant chains have not been examined and so it is not clear if the mutations affect the initial folding of the α2(VI) C-terminal region, or if they compromise the interactions that lead to chain selection and monomer formation, or if they act later in the assembly pathway and slow
dimer, tetramer, or microfibril assembly. The biosynthetic consequences of two recessive α2(VI) C2 subdomain mutations, p.N897del and p.L837P, have however, been explored. These mutations were identified in a patient with Ullrich congenital muscular dystrophy (10). Almost no collagen VI was assembled and secreted by fibroblasts from this patient suggesting that the mutations may have prevented the initial chain association event. In light of our data indicating that the critical chain recognition sequences are in the C1 subdomain, it is likely that rather than preventing chain selection, the C2 mutations cause the C2 subdomain to misfold, and the misfolded chains are recognized and degraded by intracellular quality control mechanisms and are therefore unavailable for assembly.

Formation of collagen VI dimers and tetramers also proceeded normally when the α3(VI) C2-C5 subdomains were deleted demonstrating that these regions are not directly involved in any of the intracellular collagen VI assembly events. Naturally occurring mutations in patients have revealed that dimer formation is dependent on the structure of the triple helix toward the N terminus, around residues 79–96 of the helix (4), and triple helical deletions N-terminal to this region compromise both tetramer and microfibril formation (10). Site-directed mutagenesis and in vitro transcription/translation in the presence of semi-permeabilized cells has suggested that α2(VI) triple helical residues 106–114 and the α2(VI) C2 domain are involved in the interactions leading to dimer formation (34), however, in these experiments the α2(VI) chains were synthesized without the other chains and the α3(VI) chain did not form dimers and tetramers even when the critical α2(VI) triple helical and C2 domains were inserted. Further studies using all three chains in intact cells will be required to resolve this discrepancy and confirm the involvement of these α2(VI) sequences in dimer formation.

The protein domains and sequences important for the end-to-end association of tetramers into microfibrils are also only partially understood. Predicted models of the junctional complex of collagen VI microfibrils are based on sequence data and the structures observed by electron microscopy, and suggest that the N-terminal domains of adjacent tetramers overlap, so that they are available to interact with each other, and with the C-domains and triple helix of the adjacent tetramer (2, 17). Consistent with this, tetramers from patients with glycine substitutions or in-frame deletions toward the N-terminal end of the triple helix show reduced end-to-end association of the tetramers (10, 20). In SaOS-2 stable transfection studies the α3(VI) N-terminal subdomain N5 was shown to be critical for microfibril formation (21), although its interacting partner is not known. The data presented here now shows that the α3(VI) C-terminal subdomain C5 is also critical for microfibril formation. It is possible that the interactions driving microfibril formation are between α3(VI) N5 and α3(VI) C5 in adjoining tetramers.

A number of studies have suggested that a large portion of the α3(VI) chain is cleaved from the molecule in the extracellular matrix. The α3(VI) chain synthesized by cultured cells is around 260 kDa (3, 15, 35), whereas α3(VI) chains in tissues have a maximum mass of around 200 kDa (15, 35). Bacterial collagenase digests of purified tissue-derived α3(VI) chains contain a major ~160 kDa fragment that is too large to be derived from the C-terminal globular domain, which has a predicted mass of 88 kDa (35). This indicates that most of the N-terminal globular domain is present in tissues. However, a fragment corresponding to the α3(VI) C-terminal globular domain could not be detected in these experiments, suggesting that the observed processing occurs at the C-terminal end of the chain. This proposal is supported by amino acid sequencing of tissue-derived collagen VI, which identified α3(VI) N-terminal subdomains N8, N6, and N1-N4, and the C-terminal subdomain C1, but not C2-C5 (13). More recent studies using an antibody specific for the α3(VI) C5 subdomain clearly demonstrate that C5 is present on α3(VI) chains synthesized and secreted by cultured cells, but C5 was not detected in the extracellular matrix of cultured cells or tissues (15, 27). Together, these data indicated that part of the α3(VI) C-terminal globular domain was removed at some point after synthesis; however, the function of this processing and the precise cleavage site in the C-terminal globular domain were not determined. Our observation that a small proportion of the α3(VI) chains in the medium of transfected SaOS-2 cells co-migrated with α3(VI) N6-C1 chains, suggested that the cleavage site may be between C1 and C2, and that α3(VI) processing results in chains containing only subdomain C1 at the C-terminal end. Since domains C2-C5 are ~65 kDa, this fitted well with the ~60 kDa mass difference between cell-derived and tissue-derived α3(VI) chains, however, further investigation revealed that the processed α3(VI) chains in the SaOS-2 cells were derived from free α3(VI) chains that had not assembled into functional tetramers. While this suggests that the processing event is unlikely to be physiological, it does indicate that the α3(VI) chain is susceptible to proteolytic cleavage between subdomains C1 and C2.

Our data showing that the α3(VI) C5 subdomain is critical for microfibril formation, together with the evidence that the C-terminal end of the α3(VI) chain is not part of the mature collagen VI microfibrils, raised the possibility that cleavage within the α3(VI) C-terminal globular domain may be an obligatory step in microfibril formation, in the same way that cleavage of the collagen I C-propeptide is essential for fibrillogenesis (36). However, to our surprise, immunostaining showed that the C5 domain was present in the extracellular matrix of both SaOS-2 α3(VI) N6-C5 expressing cells, and normal human fibroblasts. This finding directly contradicts an earlier study that found that the same C5 antibody did not stain the extracellular matrix of human fibroblasts (15). The reason for this discrepancy is unknown. Our data are, however, consistent with the reported presence of the C5 domain in the newly formed collagen VI microfibrils in the immediate pericellular matrix of human cartilage (27), and we were able to replicate this finding in human cartilage. The mature microfibrils of the outer pericellular matrix of chondrocytes lack this domain (27), and it also could not be detected in a range of other human tissues (15). Thus, while the α3(VI) C5 domain is critical for collagen VI microfibril formation, cleavage of C5 is not essential for microfibril formation. Maturation of collagen VI microfibrils in the extracellular matrix appears to involve one or more proteolytic events that remove the C-terminal end of the α3(VI) chain. Differential processing of this domain has the potential to regulate protein-protein interactions in the extracellular matrix and thus modulate the function of the collagen VI microfibrils.

REFERENCES

1. Timpl, R., and Engel, J. (1987) in Structure and Function of Collagen Types (Mayne, R., and Burgess, R. E., eds) pp. 105–143, Academic Press, Orlando, FL.
2. Timpl, R., and Chu, M.-L. (1994) in Extracellular Matrix Assembly and Structure (Yurchenco, P. D., Birk, D., and Mecham, R. P., eds), pp. 207–242, Academic Press, Orlando.
3. Lamande, S. R., Bateman, J. F., Hutchinson, W., McKinlay Gardner, R. J., Bewer, S. P., Byrne, E., and Dahl, H. H. (1998) Hum. Mol. Genet. 7, 981–989.
4. Lamande, S. R., Shields, K. A., Kornberg, A. J., Shield, L. K., and Bateman, J. F. (1999) J. Biol. Chem. 274, 21817–21822.
5. Jobis, G. J., Keizers, H., Vreijling, J.-P., de Visser, M., Speer, M. C., Wolterman, R. A., Baas, F., and Bolhuis, P. A. (1996) Nat. Genet. 14, 113–115.
6. Pan, T.-C., Zhang, R.-Z., Pericak-Vance, M. A., Tandan, R., Fries, T., Stajich, J. M., Viles, K., Vance, J. M., Chu, M.-L., and Speer, M. C. (1998) Hum. Mol. Genet. 7, 807–812.
7. Pepe, G., Giusti, B., Bertini, E., Brunelli, T., Saitta, B., Corneglio, P., Bolognese, A., Merlini, L., Federici, G., Abbate, R., and Chu, M.-L. (1999) Biochem. Biophys. Res. Commun. 258, 802–807.
8. Vanegas, O. C., Bertini, E., Zhang, R.-Z., Petretti, S., Minosse, C., Sabatelli, P., Giusti,
C-terminal α3(VI) Subdomains and Collagen VI Assembly

B., Chu, M.-L., and Pepe, G. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 7516–7521
9. Demir, E., Sabatelli, P., Allamand, V., Ferreiro, A., Moghadaszedeh, B., Makrelouf, M., Topaloglu, H., Echenne, B., Merlini, L., and Guicheney, P. (2002) Am. J. Hum. Genet. 70, 1446–1458
10. Baker, N. L., Morgelin, M., Peat, R., Goemans, N., North, K. N., Bateman, J. F., and Lamande, S. R. (2005) Hum. Mol. Genet. 14, 279–293
11. Pan, T. C., Zhang, R. Z., Sudano, D. G., Marie, S. K., Bonnemann, C. G., and Chu, M. L. (2003) Am. J. Hum. Genet. 73, 355–369
12. Chu, M. L., Pan, T. C., Conway, D., Kuo, H. J., Glanville, R. W., Timpl, R., Mann, K., and Deutzmann, R. (1989) EMBO J. 8, 1939–1946
13. Chu, M. L., Zhang, R. Z., Pan, T. C., Stokes, D., Conway, D., Kuo, H. J., Glanville, R., Mayer, U., Mann, K., Deutzmann, R., and Timpl, R. (1990) EMBO J. 9, 385–393
14. Specks, U., Mayer, U., Nischt, R., Specks, U., Pan, T. C., Chu, M. L., and Timpl, R. (1994) Eur. J. Biochem. 225, 573–580
15. Colombatti, A., Bonaldo, P., Ainger, K., Bressan, G. M., and Volpin, D. (1987) J. Biol. Chem. 262, 14454–14460
16. Engel, J., Furthmayr, H., Odermatt, E., von der Mark, H., Aumailler, M., Fleischmaier, R., and Timpl, R. (1985) Ann. N. Y. Acad. Sci. 466, 25–37
17. Furthmayr, H., Odermatt, H., Timpl, R., Odermatt, E., and Engel, J. (1983) Biochem. J. 211, 303–311
18. Odermatt, E., Risteli, J., van Delden, V., and Timpl, R. (1983) Biochem. J. 211, 295–302
19. Lamande, S. R., Morgelin, M., Selan, C., Jobsis, G. J., Baas, F., and Bateman, J. F. (2002) J. Biol. Chem. 277, 1949–1956
20. Fitzgerald, J., Morgelin, M., Selan, C., Wiberg, C., Keene, D. R., Lamande, S. R., and Bateman, J. F. (2001) J. Biol. Chem. 276, 187–193
21. Horton, R. M., and Pease, L. (1991) in Directed Mutagenesis (McPherson, M. J., ed), pp. 217–247, IRL Press, Oxford
22. Lamande, S. R., Sigalas, E., Pan, T. C., Chu, M. L., Dziadek, M., Timpl, R., and Bateman, J. F. (1998) J. Biol. Chem. 273, 7423–7430
23. Engvall, E., Hesse, H., and Kler, G. (1986) J. Cell Biol. 102, 703–710
24. Hesse, H., and Engvall, E. (1984) J. Biol. Chem. 259, 3955–3961
25. Bateman, J. F., Masca, R., Chan, D., and Cole, W. G. (1984) Biochem. J. 217, 103–115
26. Aigner, T., Hambach, L., Soder, S., Schluter-Schreberd, U., and Poschl, E. (2002) Biochem. Biophys. Res. Commun. 290, 743–748
27. Ball, S. G., Balock, C., Kielby, C. M., and Shuttleworth, C. A. (2001) J. Biol. Chem. 276, 7422–7430
28. Scachetti, T., Gilland, E. M., Subramony, S. H., Vedanarayanan, V., Crowe, C. A., Thakore, N., Binger, M., and Hoffman, E. P. (2002) Neurology 58, 593–602
29. Lampe, A. K., Duan, D. M., von Niederhausern, A. C., Hamil, C., Aoyagi, A., Laval, S. H., Marie, S. K., Chu, M. L., Swoboda, K., Muntoni, F., Bonnemann, C. G., Flanigan, K. M., Bushby, K. M., and Weiss, R. B. (2005) J. Med. Genet. 42, 108–120
30. Lampe, A. K., and Bushby, K. M. (2005) J. Med. Genet. 42, 675–685
31. Nagy, E., and Maquat, L. E. (1998) Trends Biochem. Sci. 23, 198–199
32. Ishikawa, H., Sugie, K., Murayama, K., Awa, A., Suzuki, Y., Noguchi, S., Hayashi, Y. K., Nonaka, I., and Nishino, I. (2004) Neurology 62, 620–623
33. Ball, S., Bella, J., Kielby, C., and Shuttleworth, A. (2003) J. Biol. Chem. 278, 15326–15332
34. Trub, B., and Winterhalter, K. H. (1986) EMBO J. 5, 2815–2819
35. Kallal, E. D., Hulmes, D. J., Hojima, Y., and Prockop, D. J. (1990) Ann. N. Y. Acad. Sci. 580, 214–224