The N-terminal N5 Subdomain of the α3(VI) Chain Is Important for Collagen VI Microfibril Formation*

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Collagen VI assembly is unique within the collagen superfamily in that the α1(VI), α2(VI), and α3(VI) chains associate intracellularly to form triple helical monomers, and then dimers and tetramers, which are secreted from the cell. Secreted tetramers associate end-to-end to form the distinctive extracellular microfibrils that are found in virtually all connective tissues. Although the precise protein interactions involved in this process are unknown, the N-terminal globular regions, which are composed of multiple copies of von Willebrand factor type A-like domains, are likely to play a critical role in microfibril formation, because they are exposed at both ends of the tetramers. To explore the role of these subdomains in collagen VI intracellular and extracellular assembly, α3(VI) cDNA expression constructs with sequential N-terminal deletions were stably transfected into SaOS-2 cells, producing cell lines that express α3(VI) chains with N-terminal globular domains containing modules N9-N1, N6-N1, N5-N1, N4-N1, N3-N1, or N1, as well as the complete triple helix and C-terminal globular domain (C1-C5). All of these transfected α3(VI) chains were able to associate with endogenous α1(VI) and α2(VI) to form collagen VI monomers, dimers, and tetramers, which were secreted. Importantly, cells that expressed α3(VI) chains containing the N5 subdomain, α3(VI) N9-C5, N6-C5, and N5-C5, formed microfibrils and deposited a collagen VI matrix. In contrast, cells that expressed the shorter α3(VI) chains, N4-C5, N3-C5, and N1-C5, were severely compromised in their ability to form end-to-end tetramer assemblies and failed to deposit a collagen VI matrix. These data demonstrate that the α3(VI) N5 module is critical for microfibril formation, thus identifying a functional role for a specific type A subdomain in collagen VI assembly.

Although collagen VI is expressed in virtually all connective tissues, where it forms a complex and extensive microfibrillar network (see Refs. 1 and 2 for reviews), a definition of the precise functional roles of the collagen VI microfibrils in development and matrix architecture has been elusive. Even though the cell adhesion and extracellular matrix protein binding capabilities of collagen VI have suggested that it may play an important role in the interconnection between the cell and the structural scaffolding of the extracellular matrix, it is only recently that one such critical tissue-specific role in muscle has been demonstrated by the detection of mutations in collagen VI genes in Bethlem myopathy (3–6) and by the generation of a myopathy by targeted inactivation of Collα1 in mice (7).

Collagen VI is composed of three genetically distinct α-chain subunits, α1(VI), α2(VI), and α3(VI), each of which contains a relatively short triple helix and N- and C-terminal globular regions. Recent studies have demonstrated that initial association of all three chains into a collagen VI triple helical heterotrimer is a prerequisite for further intracellular assembly, stability and secretion (8). The α1(VI) and α2(VI) chains are similar in size (1009 and 998 amino acids, respectively) and contain one N- and two C-terminal subdomains of approximately 200 amino acids that show homology to the type A domains of von Willebrand factor (vWF) (9). In contrast, the α3(VI) chain is much larger (up to 3149 amino acids), containing 6 to 10 N-terminal vWF type A-like subdomains (depending on alternative splicing), two C-terminal vWF type A-like subdomains, and subdomains similar to type III fibronectin repeats, and Kunitz protease inhibitors (10, 11).

The precise protein interactions initiating and regulating formation of the unique collagen VI microfibril supramolecular assemblies are not known, but elegant structural studies have identified the organization of assembly intermediates, providing a model for a hierarchical assembly process unlike that of any other members of the collagen family (12, 13). Following heterotrimeric assembly of the α1(VI), α2(VI), and α3(VI) chains, these collagen VI monomers form higher order structures intracellularly by aligning in an anti-parallel manner with a 30-nm stagger, first to form dimers and then tetramers by lateral association of dimers. After secretion, the tetramers associate end-to-end to form microfibrils with a distinctive 100-nm periodicity, comprising beaded globular domains separated by short triple helical regions (12, 13). However, the critically important questions of what protein structures and interactions drive these complex and specific associations during collagen VI assembly have not been addressed in detail.

It is likely that numerous motifs within the collagen VI α-chains contribute to the final microfibril structure and to the development of its heterotypic interactions with other matrix components. The type A subdomains present in all three collagen VI subunits, and found in a number of other extracellular proteins including integrins, matrilins, complement components, and collagens VII, XII, and XIV (14–16), are likely to

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1 The abbreviations used are: vWF, von Willebrand factor; PCR, polymerase chain reaction; PBS, phosphate-buffered saline.
play an important role, as they have been shown to be commonly involved in specific molecular interactions (14). In particular, it seems probable that the N terminus of the α3(VI) chain may be the crucial domain involved in tetramer-tetramer association because its multiple type A modules would be exposed at both ends of the tetramers during microfibrillar formation.

To examine systematically the role of the α3(VI) N-terminal type A subdomains, N9 to N1, in collagen VI intracellular tetramer assembly and extracellular microfibrillar formation α3(VI) cDNA constructs with sequential deletions of the N-terminal type A modules were stably transfected into SaOS-2 cells, producing a range of cell lines that express α3(VI) chains with N-terminal domains containing modules N9-N1, N6-N1, N5-N1, N4-N1, N3-N1, or N1, as well as the complete triple helix and C-terminal globular domain. Our data show that α3(VI) subdomains N2 to N9 are not required for intracellular heterotrimer assembly or for the formation of disulfide-bonded dimers and tetramers. Ultrastructural examination of the secreted collagen VI demonstrated that tetramers were able to associate end-to-end to form microfibrils in cells expressing α3(VI) chains containing the N-terminal N9-N1, N6-N1, and N5-N1 modules. Significantly, however, the efficiency of tetramer-tetramer association was reduced in cells expressing α3(VI) chains with N-terminal domains lacking the N5 module. These data provide the first evidence that an α3(VI) N-terminal subdomain (N5) plays a critical role in the interactions between collagen VI tetramers leading to the formation of the microfibrillar network.

**EXPERIMENTAL PROCEDURES**

Production of α3(VI) cDNA Expression Constructs—All constructs were cloned in the mammalian expression vector pCMV (Invitrogen), which contains the neomycin phosphotransferase gene conferring resistance to G418. Production and stable expression of the α3(VI) N6-C5 construct, encoding the BM40 signal sequence and the α3(VI) protein domains N6 to C5 (Fig. 1), have been described previously (8). Constructs encoding α3(VI) domains N5-C5, N4-C5, and N1-C5 were generated by PCR using the α3(VI) N6-C5 cDNA as a template. Forward primers were designed to anneal at the 5′ end of subdomain N5, N4, and N1, beginning at nucleotides 2710 (domains N9-C5, ATG at base 256 (10)), 3317, and 5131, respectively, and each contained an NheI site at the 5′ end to allow cloning of the PCR product into the NheI site at the 3′ end of the BM40 signal sequence of pCMV α3(VI) N6-C5 (8). The downstream primer in each reaction corresponded to nucleotides 5748–5771 near the 5′ end of the triple helix and included the XhoI site at base 5757. PCR was performed using the proof-reading polymerase Pfu (Stratagene). The resultant fragments were digested with NheI and SacI and used to replace the corresponding fragment of the α3(VI) N6-C5 construct.

The α3(VI) N3-C5 construct was generated by digesting α3(VI) N6-C5 with NheI and Bpu1 1021 recognition site (base 3778). The restriction enzyme-generated overhangs were filled in using Klenow polymerase (17), and the resulting blunt ends were religated. The Bpu1 1021 site is in the N4 subdomain, and therefore the α3(VI) N3-C5 construct contains 151 nucleotides of N4 at the 5′ end.

To facilitate production of the α3(VI) N9-C5 construct, an NheI site within the N9 subdomain cDNA was deleted using strand overlap extension PCR (18) to introduce a silent T to C substitution at position 747. Primers A and B were used to amplify bases 351–755 using the cDNA clone FO19 (10) as a template, and primers C and D to amplify bases 753–2097. Primers B and C overlapped, and both contained the single nucleotide substitution. To allow cloning, primer A contained an NheI site at the 5′ end. These amplification products were gel-purified, and 100 ng of each used was as the template in a second round of overlapping PCR with primers A and D. The resulting PCR product was digested with NheI and cloned into the NheI site of α3(VI) N6-C5 to generate the α3(VI) N9-C5 construct.

To ensure that no errors had been introduced during PCR and cloning, all constructs were transcribed and translated in vitro (TNT<sup>®</sup>, Promega), and the regions generated by PCR were sequenced (Amplitag<sup>®</sup>, PerkinElmer Life Sciences) (data not shown).

**Cell Culture and Transfections**—The SaOS-2 human osteosarcoma cell line (ATCC HTB-85), which expresses α1(VI) and α2(VI) mRNA but not α3(VI) mRNA (8), was maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum as described previously (19). Cells were transfected with the α3(VI) expression constructs using LipofectAMINE<sup>®</sup> reagent (Life Technologies, Inc.). Stably transfected cells were selected in medium containing 500 μg/ml G418 (Life Technologies, Inc.) and individual clones isolated and expanded into cell lines.

**Northern Blot Analysis**—RNA was isolated from cultured SaOS-2 cells using RNeasy<sup>®</sup> (Qiagen). Total RNA (5 μg) was analyzed under denaturing conditions on 1% agarose gels and transferred to nitrocellulose membranes (17). Filters were hybridized to [α<sup>32</sup>P]-labeled α3(VI) probe, and α3(VI) probe (P1), α3(VI) probe (P2), and α3(VI) probe (P3) were washed, and specific hybridization was visualized by autoradiography.

**Collagen VI Biosynthetic Labeling and Analysis**—SaOS-2 cells were grown to confluence in 10-cm<sup>2</sup> dishes, incubated overnight in the presence of 0.25 mM sodium ascorbate, and then biosynthetically labeled for 18 h with 100 μCi/ml [14C]methionine (Tran3<sup>®</sup>-label<sup>®</sup> 1032 Ci/mmol, ICN Pharmaceuticals, Inc.) in 750 μl of methionine-free and serum-free formulation. Cells were labeled using 0.02% sodium ascorbate to promote G418-resistant colonies. Production and stable expression of the modified Eagle's medium containing 10% fetal calf serum as described previously (19). Cells were transfected with the α3(VI) monoclonal antibody 3C4 obtained from Dr E. Engvall (21) or a polyclonal collagen VI antibody (Life Technologies, Inc.) and 100 μl of 20% protein A-Sepharose. Immunoprecipitated complexes were washed twice with 50 mM Tris/HCl, pH 7.5, containing 5 mM EDTA, 0.15 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 20 mM N-ethylmaleimide, and 5 mM EDTA. The cell layer was solubilized in 50 mM Tris/HCl, pH 7.5, containing 5 mM EDTA, 0.15 mM NaCl, 1% Nonidet P-40, and 1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, 20 mM N-ethylmaleimide, and 5 mM EDTA. The cell layer was solubilized in 50 mM Tris/HCl, pH 7.5, containing 0.15 mM NaCl, 5 mM EDTA, and 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-polyacrylamide gel electrophoresis on 5% (w/v) polyacrylamide gels. Collagen VI triple helical monomers, dimers, and tetramers were analyzed on 4% (w/v) acrylamide/0.5% (w/v)-agarose composite gels under nonreducing conditions (5, 6, 8). Radioactively labeled proteins were detected by fluorography (19) or imaged using a PhosphorImager (Molecular Dynamics, STORM<sup>®</sup>).
expressed on 5% polyacrylamide gels under reducing conditions to visualize medium fractions immunoprecipitated with collagen VI antibodies used for immunoprecipitation were specific for the a3 helix and C-terminal domains intact.

A

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\begin{array}{c}
N1 \quad C1 \quad C2 \\
N1 \quad C1 \\
N1 \\
\end{array}
\]

B

\[
\begin{array}{c}
N9 \quad N5 \\
N6 \quad N6 \\
N4 \quad N5 \\
N3 \quad N3 \\
N1 \quad N1 \\
\end{array}
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Collagen VI Intracellular Assembly in Transfected SaOS-2 Cells—To examine the contribution of the a3(VI) N-terminal globular subdomains to collagen VI intracellular assembly, microfibril formation, and matrix deposition, we stably transfected SaOS-2 cells, which express a1(VI) and a2(VI) mRNA and protein but no a3(VI) mRNA (8), with six a3(VI) cDNA constructs from which the N-terminal globular domains N9 to N1 were progressively deleted. As a result of alternative splicing, more than 95% of the a3(VI) mRNAs found in cells and tissues lack the N10 subdomain (11); therefore, the a3(VI) N9-C5 construct was used as the “full-length” control in this study. Individual cell clones transfected with either the control construct (a3(VI) N9-C5) or the shorter constructs (a3(VI) N6-C5, N5-C5, N4-C5, N3-C5, and N1-C5) (Fig. 1) were selected in medium containing G418 and screened for expression of a3(VI) mRNA by Northern blotting (data not shown). Cell lines were metabolically labeled with [35S]methionine and cell layer and medium fractions immunoprecipitated with collagen VI antibodies. The immunoprecipitated material was electrophoresed on 5% polyacrylamide gels under reducing conditions to visualize the individual collagen VI chains. All transfected cell lines expressed a3(VI) protein that migrated with an apparent molecular mass in good agreement with the predicted size of each of the shorter chains (Fig. 2) when compared with the control a3(VI) N9-C5 (Fig. 2, lanes 11 and 12). Because the collagen VI antibodies used for immunoprecipitation were specific for the a3(VI) chain (N9, N6, N5, N4, and N3 constructs), or assemblies of all three chains (N1 construct), co-immunoprecipitation of a1(VI) and a2(VI) chains in all of the cell lines indicated that the shortened a3(VI) chains had formed collagen VI heterotrimers that were able to be secreted. Collagen VI found in the medium had a slower electrophoretic mobility compared with that isolated from the cell layer fraction. This is a consistent finding in both transfected SaOS-2 cells and primary human fibroblasts (5, 6, 8), and although the reason for this size difference is not known, it may reflect differences in glycosylation between secreted collagen VI and the more recently synthesized intracellular collagen VI.

Secretion of all three collagen VI chains by each of the transfected cell lines provided indirect evidence that heterotrimers containing the shortened a3(VI) chains were able to assemble further into tetramers, the normal secreted form of collagen VI (22). Tetramer formation was demonstrated directly by composite agarose/acylamide gel electrophoresis under nonreducing conditions (Fig. 3). The predominant form of collagen VI secreted by cells expressing control a3(VI) N9-C5 chains migrated with a molecular mass of approximately 2000 kDa (Fig. 3, lane 12) as expected for collagen VI tetramers. Comparable results were seen in cultures expressing each of the shortened a3(VI) chains (Fig. 3, lanes 1-10), clearly demonstrating that the a3(VI) N-terminal subdomains N9-N2 are not required for collagen VI monomer, dimer, and tetramer formation and secretion.

Collagen VI Microfibril Formation—To determine whether collagen VI tetramers containing shortened a3(VI) chains could self-associate to form end-to-end assemblies of tetramers, secreted collagen VI was examined by negative staining electron microscopy. Medium from cells expressing a3(VI) N9-C5, a3(VI) N6-C5, and a3(VI) N5-C5 (Fig. 4A) contained microfibrils composed of multiple tetramers as well as single tetramers. In contrast, the majority of the collagen VI in medium from cells expressing the shorter a3(VI) chains, a3(VI) N4-C5, a3(VI) N3-C5, and a3(VI) N1-C5 (Fig. 4B), was present as single tetramers. Microfibril formation in multiple clonal cell lines expressing the control and shortened a3(VI) chains was quantified by analysis of a large number of micrograph fields (Fig. 5). The occurrence of “microfibrils” containing 1–7 tetramers is shown as a percentage of the total number of microfibrils. In cells expressing a3(VI) N9-C5, N6-C5, and N5-C5, 70–80% of the microfibrils contained 2–7 tetramers and only 20–30% were single tetramers (Fig. 5, A–C). In striking contrast, in medium from cells expressing a3(VI) N4-C5, N3-C5, and N1-C5, more than 70% of all microfibrils were single tetramers, with only a small number of double tetramers and virtually no higher aggregations of tetramers (Fig. 5, D–F). The efficiency of microfibril formation was comparable in all of the clonal cell lines expressing a3(VI) N9-C5, N6-C5, and N5-C5 (Fig. 5, A–C) and was reduced in all of the cell lines expressing a3(VI) N4-C5, N3-C5, and N1-C5 (Fig. 5, D–F). These data demonstrate that all of the cells expressing a3(VI) chains, including the N5 subdomain, are able to form microfibrils with similar efficiency, whereas those lacking the N5 have compromised multimerization, suggesting that the a3(VI) N5 module is crucial for the interactions between tetramers that lead to the formation of microfibrils.

To formally exclude the possibility that the efficiency of tetramer aggregation was dependent on the concentration of tetramers in the medium, the levels of a3(VI) mRNA and protein in an a3(VI) N5-C5 cell line (able to form microfibrils)
and two α3(VI) N3-C5 cell lines (not able to form microfibrils) were examined. Northern blot analysis demonstrated that the α3(VI) N5-C5 cell line (clone 28) contained much less α3(VI) mRNA than either of the two α3(VI) N3-C5 cell lines (clones 19 and 17) (Fig. 6A). The amount of collagen VI protein produced by these clones was consistent with the level of expression of Fig. 2. Electrophoretic analysis of collagen VI. Transfected SaOS-2 cells were metabolically labeled for 18 h with [35S]methionine, and collagen VI in the cell (C) and medium (M) fractions were immunoprecipitated and resolved on 5% polyacrylamide gels under reducing conditions. Collagen VI α1(VI), α2(VI), and α3(VI) subunits are indicated on the right, and the migration position of the [14C]-methylated 200-kDa standard is shown on the left.

Fig. 3. Analysis of collagen VI tetramer assembly. Transfected SaOS-2 cells were labeled biosynthetically for 18 h, and collagen VI was immunoprecipitated from the cell (C) and medium (M) fractions electrophoresed on 2.4% (w/v) acrylamide, 0.5% (w/v) agarose composite gels under nonreducing conditions to visualize collagen VI tetramers. Collagen VI tetramers are indicated on the right, and the migration position of the unreduced laminin marker (900 kDa) is shown on the left.

Fig. 4. Negative staining electron microscopy of secreted collagen VI. 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, adsorbed onto carbon-coated grids, stained with uranyl formate, and examined by electron microscopy. Media from cells expressing α3(VI) N5-C5 (A), N6-C5, and N9-C5 contained collagen VI tetramers that had associated end-to-end to form microfibrils. In media samples from α3(VI) N1-C5 (B), N3-C5, and N4-C5, the greater portion of the collagen VI was present as single tetramers.

Fig. 5. Quantitative analysis of collagen VI tetramer-tetramer association. Collagen VI secreted into the medium of transfected SaOS-2 cells was visualized by negative staining electron microscopy, and the ability of the tetramers to associate end-to-end was quantitated. The occurrence of microfibrils containing 1–7 tetramers is shown as a percentage of the total number of microfibrils. When available, multiple clonal cell lines expressing each α3(VI) cDNA construct were analyzed. SaOS-2 cells expressing: A, α3(VI) N9-C5; B, α3(VI) N6-C5; C, α3(VI) N5-C5; D, α3(VI) N4-C5; E, α3(VI) N3-C5; F, α3(VI) N1-C5.
**DISCUSSION**

In this study, we investigated the contribution of α3(VI) N-terminal vWF type A-like subdomains N2 to N9 to collagen VI intracellular assembly, secretion, and microfibril formation. We show that the α3(VI) subdomains N2 to N9 are not necessary for collagen VI intracellular triple helical heterotrimer assembly, the subsequent formation of disulfide-bonded dimers and tetramers, and secretion of the collagen VI tetramers from the cell. However, the N-terminal α3(VI) type A modules were found to play a critical extracellular role in the end-to-end association of tetramers to form collagen VI microfibrils. Cells that expressed α3(VI) chains containing the N5 subdomain, α3(VI) N5-C5, α3(VI) N6-C5, and α3(VI) N9-C5, had the ability to form collagen VI microfibrils and deposit a collagen VI ma-

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**FIG. 6. Relative collagen VI mRNA and protein expression in SaOS-2 cells.** A, Northern blot analysis of collagen VI mRNA expression. Total RNA from untransfected SaOS-2 cells (lane 1) and from cells expressing α3(VI) N5-C5 (clone #28, lane 2) or α3(VI) N3-C5 chains (clones #19 and #17, lanes 3 and 4) was separated on a denaturing agarose gel, transferred to nitrocellulose, and hybridized simultaneously with 32P-labeled α1(VI), α2(VI), and α3(VI) cDNAs. Migration of α1(VI), α2(VI), and α3(VI) mRNAs are indicated on the right. B, Electrophoretic analysis of collagen VI protein expression. Untransfected SaOS-2 cells and cells transfected with the α3(VI) N5-C5 and N3-C5 constructs were labeled metabolically for 18 h with [35S]methionine and collagen VI in the cell (C) and medium (M) fractions immunoprecipitated and electrophoresed on a 5% polyacrylamide gel under reducing conditions. Collagen VI α1(VI), α2(VI), and α3(VI) subunits are indicated on the right.

α3(VI) mRNA. Small amounts of collagen VI protein were present in the cell and medium fractions of the α3(VI) N5-C5 line (Fig. 6B, lanes 3 and 4), and substantially larger amounts were produced by the two α3(VI) N3-C5 clones (lanes 5–8). The faint bands in lanes 3 and 4 were clearly identified as collagen VI following a longer exposure. Because mRNA and protein expression is higher in the two α3(VI) N3-C5 cell lines than in the α3(VI) N5-C5 line, and end-to-end tetramer assembly is more efficient in the α3(VI) N5-C5 line than in either of the two α3(VI) N3-C5 cell lines (Fig. 5), it is clear that the efficiency of tetramer aggregation is not related to the level of collagen VI synthesis and the concentration of tetramers available for microfibril formation.

To determine which cell lines were able to incorporate collagen VI tetramers into an extracellular matrix, transfected SaOS-2 cells were grown for 15 days post-confluence in the presence of 0.25 mM sodium ascorbate and stained with a collagen VI antibody. Bound antibody was detected with fluorescein isothiocyanate-conjugated sheep anti-mouse Ig. All images are X40.

**Fig. 7. Collagen VI in the in vitro accumulated extracellular matrix.** SaOS-2 cells transfected with α3(VI) N9-C5 (A), α3(VI) N6-C5 (B), α3(VI) N5-C5 (C), and α3(VI) N4-C5 (D) were grown for 15 days post-confluence in the presence of 0.25 mM sodium ascorbate and stained with a collagen VI antibody. Bound antibody was detected with fluorescein isothiocyanate-conjugated sheep anti-mouse Ig. All images are X40.
The subsequent complex hierarchical process of collagen VI intracellular assembly, the formation of dimers and tetramers, does critically involve the participation of collagen VI triple-helix sequences. Ultrastructural and binding studies predict that a specific interaction between the C-terminal globular domain of one monomer and the triple helix of the adjacent overlapping antiparallel monomer is involved in dimer formation, an interaction that is stabilized by disulfide bonding (12, 13, 28). Tetramers are thought to be stabilized by disulfide bonds between the N-terminal ends of the triple helix of the dimers (12, 13). Furthermore, collagen VI heterotrimers, containing deletions that disturb the structure of the helix, are unable to assemble further into disulfide-bonded dimers and tetramers, and molecules containing the mutant chain are not secreted (5).

The structural models proposed for the supramolecular organization of collagen VI tetramers into microfibrils (9, 12, 13) provide us with a basis for understanding the importance of the type A domains in microfibril formation. The lateral association of dimers into tetramers generates a structure in which the type A domains from the N termini of all three α-chains are exposed at both ends of the tetramer. The model for the junctional complex further predicts that N-terminal domains of adjacent tetramers overlap in this region, such that they are available to interact with each other and with the C-domains or the helical domain of the adjacent tetramer, providing multiple potential sites for the noncovalent interactions that stabilize microfibrils (9, 12, 13). Although the N termini in this junctional complex contain multiple type A subdomains from the α1(VI), α2(VI), and α3(VI) chains, all of which could potentially function in these interactions, our data demonstrates that the α3(VI) N5 subdomain is critical for microfibril formation. The α3(VI) chain is expressed as a number of splice variants in vivo and in cultured cells in which specific N-terminal modules are deleted from the protein structure (10, 11, 29). It is of interest to note that the N5 module is present in all of these splice variants, consistent with a crucial role for N5 in the formation of microfibril assembly competent collagen VI. Although it is clear that the N5 module is important for extracellular tetramer association, other subdomains C-terminal to N5, and/or those within α1(VI) or α2(VI) and/or the N-terminal region of the triple helix, could contribute to microfibril formation or stability by providing an interaction partner for N5. A minor role for other subdomains is also suggested by the small amount of end-to-end association that occurs between tetramers lacking N5.

There are several other examples of interactions between A domains, either within the same molecule or between different extracellular matrix components, suggesting that these protein modules may play an important role in matrix architecture and assembly. Matrilin 1 contains two vWF type A-like domains, which have been shown to be involved in the assembly of matrilin oligomers and in the formation of matrilin-1 filamentous networks, suggesting that interactions between matrilin-1 type A domains can occur (30). Interaction between type A domains from different matrix proteins have been demonstrated for the A1 and A3 domains of vWF, both of which associate with the globular regions of chicken collagen VI microfibrils to promote the adhesion of platelets to collagen VI at high shear forces (31).

Further evidence for the role of α3(VI) N-terminal subdomains in the formation of functional collagen VI comes from studies of a family with Bethlem myopathy in which the mutation is a glycine to glutamate change at amino acid 167 of the

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2 Marie Dziadek, personal communication.
α3(VI) N2 subdomain (4). Normal levels of all three chains were detected in the medium of fibroblasts from affected individuals, suggesting that monomer and tetramer formation was not impaired (4). This result is consistent with our data showing that the removal of the entire N2 subdomain, as with the α3(VI) N1-C5 construct, does not affect monomer and tetramer formation and secretion. The molecular mechanism of how this N2 mutation results in a clinical phenotype is not known, but it could perturb collagen VI function by structurally interfering with end-to-end association of tetramers or, possibly, by modifying interactions of the mutant collagen VI with other matrix components.

Our emerging understanding of collagen VI assembly is that the α3(VI) N-terminal domain plays at least two broad roles, in microfibril assembly and in collagen VI-matrix interactions. The N-terminal domain of the α3(VI) chain interacts with vWF and heparin in vitro. The A1 subdomain of vWF interacts with the chicken α3(VI) N8 (31), and heparin binding has been assigned to α3(VI) subdomains N3, N6, and N9 suggesting the potential for binding to heparan sulfate proteoglycans (32). It seems reasonable to speculate that the specific role of each A-type subdomain may be related to its location within the three-dimensional structure of the globular beads of the tetramer junctional complex. Our data would predict that the N5 domain must be positioned so that it is exposed on the interacting surface in intimate contact with the N- and/or C-terminal domains or the triple helix of the collagen VI in the adjacent tetramer. Other subdomains may be positioned so as to be available to participate in interactions with a variety of extracellular matrix proteins.

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