Type VI Collagen Anchors Endothelial Basement Membranes by Interacting with Type IV Collagen*

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Type VI collagen filaments are found associated with interstitial collagen fibers, around cells, and in contact with endothelial basement membranes. To identify type VI collagen binding proteins, the amino-terminal domains of the α1(VI) and α2(VI) chains and a part of the carboxy-terminal domain of the α3(VI) chain were used as bait in a yeast two-hybrid system to screen a human placenta library. Eight persistently positive clones were identified, two coding the known matrix proteins fibronectin and basement membrane type IV collagen and the rest coding new proteins. The amino-terminal domain of α1(VI) was shown to interact with the carboxy-terminal globular domain of type IV collagen. The specificity of this interaction was further studied using the yeast two-hybrid system in a one-on-one format and confirmed by using isolated protein domains in immunoprecipitation, affinity blots, and enzyme-linked immunosorbent assay-based binding studies. Co-distribution of type VI and type IV collagens in human muscle was demonstrated using double labeling immunofluorescent microscopy and immunoelectron microscopy. The strong interaction of type VI collagen filaments with basement membrane collagen provided a possible molecular pathogenesis for the heritable disorder Bethlem myopathy.

Type VI collagen filaments are ubiquitous. They are present in all connective tissues that contain type I and type III collagen fibers and in cartilage, a tissue that contains predominantly type II collagen. The major functions that have been suggested for type VI collagen filamentous networks are as a substrate for cell attachment and as an anchoring meshwork that connects collagen fibers, nerves, and blood vessels to the surrounding matrix (1, 2). This implies that not only is there an interaction, either direct or indirect, with the type I/III collagen fibers but also that there is an interaction with components of endothelial basement membranes.

Matrix components that have been shown to interact with type VI collagen in vitro include proteoglycans, collagens, hyaluronan, heparin, and integrins.

Proteoglycans appear to be particularly important, since cell surface-, basement membrane-, and collagen fibril-associated proteoglycans have all been reported to bind to various forms of type VI collagen. Decorin is a small dermanatan sulfate proteoglycan that binds to fibrillar collagens (3). It was shown that the leucine-rich module of the core protein bound to type VI and that the binding could be inhibited by the core proteins of fibromodulin and biglycan (4). The cell surface-associated membrane chondroitin sulfate proteoglycan NG2 was originally detected in cells from the rodent central nervous system but subsequently was also detected in blood vessels and cartilaginous structures of the head, neck, and spine. It interacts via its core protein with type VI collagen and is thought to provide machinery for transmembrane signaling (5). Since α1β1 and α2β1 integrins also bind type VI collagen (6, 7), the cell signaling potential of this molecule would appear to be significant.

The interaction with the basement membrane proteoglycan perlecan appeared to be via the type VI collagen triple helix, since pepsin-solubilized type VI collagen, but not recombinant α1(VI) and α2(VI) globular domains, showed binding to perlecan (8). However, unlike decorin and NG2, perlecan interacts via its heparan sulfate side chains.

Recombinant segments of the amino-terminal globular domain of the α3 chain, which contain a series of von Willebrand factor A domains, were used by two groups. One group reported no binding to types I, III, and IV collagens or fibropectin (9), while the second reported specific binding to fibrillar type I collagen (2). Other experiments using pepsin-solubilized type VI collagen, which only contains a small remnant of the globular domains, showed binding to fibropectin (8), while others found no binding to fibropectin or type I and III collagens but found binding to type II (4) and XIV collagens (10). In vivo studies of GP140 (α1(VI) + α2(VI)) indicated that there was an interaction of type VI collagen with fibropectin in the pericellular matrix of fibroblast (11, 12).

Data concerning the interaction of type VI with hyaluronan are also inconsistent. Intact type VI isolated from bovine cartilage (13), fetal bovine skin (14), and recombinant α3 chain A domains (9) were reported to bind hyaluronan. Furthermore, Kielt and co-workers (15) presented evidence for hyaluronan being required as a template for the assembly of type VI collagen tetramers into filaments and for the stability of the filaments once they are formed (15). The results of Speckels et al. (9) were later withdrawn (16), and their new data and our own data indicate that hyaluronan is not involved in the assembly or stability of type VI filaments (17).

The interaction spectrum of type VI collagen is clearly very complex. The results described here indicate a newly discovered direct interaction of intact native type VI collagen with type IV collagen, a major component of basement membranes. This interaction is significant because it is the first type VI collagen-protein-protein interaction with a basement membrane component that has been characterized, and it provides a plausible explanation for the Bethlem myopathy.
pheno type, a genetic disease caused by mutations in type VI

EXPERIMENTAL PROCEDURES

Yeast Matchmaker two-hybrid kit, multiple-tissue Northern blots,

**Materials**

The abbreviations used are: PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PBS, phosphate-buffered saline.
mains were biotinylated according to the method of Hnatowich et al. (22) with minor modifications. To modify the primary amino groups of type VI collagen and its fragments, the purified samples were dissolved into 0.5 M Tris-HCl, pH 8.0, buffer containing 8 M urea, 0.2 M NaCl, and 5 mM EDTA at a final protein concentration of 0.5 mg/ml and dialyzed into 0.5 M Tris-HCl, pH 8.0, buffer containing 8 M urea, 0.2 M NaCl, and 5 mM EDTA. A 50-fold molar excess of NHS-LC-biotin was added to each sample. The samples were gently shaken for 2 h at room temperature and dialyzed against PBS, 0.05% Tween 20 to remove excess biotin.

Biotin-labeled type VI collagen tetramers and globular domains were used as soluble ligands for affinity blots (17). Type IV collagen samples were separated on 8–12% SDS-PAGE gels (23) and electrotransferred onto polyvinylidene difluoride membranes. The blotted membranes were incubated with 4% BSA or 1% nonfat dried milk in PBS for 1 h to block any nonspecific binding and then with biotinylated ligands for 3 h followed by a 2-h incubation with avidin-horseradish peroxidase. The membranes were developed with color reagent as for standard Western blots. Control membranes were developed only as Western blots using chain-specific antibodies N5-VI-2 and C1-VI-1 to type IV collagen.

Immuno precipitation—The ligands used in each experiment were type IV collagen and type VI collagen in a bacterial collagenase digest of human amnion and purified NC1 domain of type IV collagen with purified type VI collagen globular domains. Type VI collagen polyclonal antibody 4104; type VI monoclonal antibody mixture containing N3-VI-2, C1-VI-2, and C12-VI-2; or type IV collagen polyclonal antibody (Southern Biotechnology) was used to precipitate the type VI-type IV complex. Preimmune serum was used to control for nonspecific precipitation. Mixtures of the proteins were incubated in PBS at room temperature for 2 h, and then antibody was added with gentle rotation for 2 h or at 4 °C overnight. PBS-washed protein G-Sepharose (1.3, v/v) was added and mixed for another 2 h at room temperature. The antigen-protein antibody G-Sepharose complex was collected by centrifugation and washed with PBS, 0.05% Tween 20 three times (5 min each). The complex was eluted from the protein G with 0.5 M acetic acid. The identity of the interacting ligands was determined by Western blot analysis.

Binding Assay—Five micrograms of pepsin-solubilized and purified type I, II, V, and XI collagens, purified intact type VI collagen tetramers, type IV collagen NC1 domain, fibronectin, and BSA were coated onto microtiter plates at 4 °C overnight. The plate was washed with PBS containing 0.05% Tween 20, incubated with 1% BSA/PBS for 30 min followed by a 3-h incubation at room temperature with varying amounts of biotinylated intact type VI collagen tetramers (0.5–5 μg). Bound type VI collagen was detected by further incubation with avidin-horseradish peroxidase (100,000 dilution) for 2 h, three PBS/Tween 20 washes, and a 30-min incubation with Sigma 104 substrate. The absorbance was measured at 405 nm. All analyses were done in duplicate, and the values were averaged.

Indirect Immunofluorescent Staining—Human skeletal muscle was trimmed and quickly frozen in hexane with liquid nitrogen. The frozen muscle was sectioned in a ultracytome, and 5-μm sections were collected on glass slides, treated with cold (~20 °C) acetone for 5 min, and allowed to air dry at room temperature. Double immunofluorescence staining was performed at room temperature on unfixed tissue. Tissue was briefly washed with PBS (5 min, twice) incubated with normal rabbit serum followed by PBS washes (3 × 5 min), and incubated for 3 h in mixtures of type IV collagen polyclonal antibody and type VI collagen monoclonal antibody VI-1. After PBS washes, they were incubated with mixtures of anti-mouse IgG fluorescein isothiocyanate (50 × dilution) and anti-rabbit IgG Texas Red (500 × dilution) conjugates for 30 min followed by PBS washes. Sections were covered with 25% glycerol in PBS buffer and viewed under a Zeiss Axioskop fluorescent microscope.

Immunoenzyme Microscopy—The method used in this study has been described previously (24). Briefly, en bloc immunolocalization of type VI collagen in human skeletal muscle from a 16-year-old individual was accomplished by incubating the tissue overnight at 4 °C in monoclonal antibody 5C6 diluted 1:5 in PBS, rinsing extensively in PBS, and then incubating overnight at 4 °C in goat anti-mouse 5-nm colloidal gold conjugate. Immunolabeled tissue was then rinsed, fixed, dehydrated, and embedded in Spurr’s epoxy prior to ultramicrotomy and examination using a Philips 410 LS transmission electron microscope.

RESULTS

Screening with the amino-terminal domain of the α1(VI) chain identified four clones from the placenta library that coded protein domains that potentially interact with type VI collagen (Table I). Sequencing the activation domain insert of the positive yeast clones revealed that two of them were from the proteins fibronectin and type IV collagen. Clone 1N-9 contained a 1107-base pair insert that included the coding sequence for the carboxyl-terminal 50 amino acids of fibronectin (positions 2399–2449), and clone 1N-52.4 contained a 1343-base pair insert that included the coding sequence for the carboxyl-terminal 233 amino acids of the α1(IV) chain (positions 1447–1669) (25). Insert 1N-14 was from a 200-kDa protein encoded by a gene on chromosome 20 called KIAA0181 (GenBank™ accession number D80003) (26). This is one of many genes identified from a human myoblast cell line KG-1 (27). The remaining positive clone insert 1N-5 contained an new sequence. Screening with the amino-terminal domain of the α2(VI) chain identified only one positive clone, 2N-36, with a 984-base pair insert. The α3(VI) chain screening identified three positive clones, 3C-30.1, 3C-45.6, and 3C-110, all containing inserts coding new protein sequences. All inserts were further characterized by multiple tissue Northern blot analysis. The tissue distribution is shown in Table I along with the approximate mRNA sizes. The tissue distributions are quite distinctive, ranging from 3C-30.1, with an apparently placentaspecific distribution, to 1N-5, which was in all tissues tested. All inserts except 3C-30.1 hybridized to fibroblast mRNA.

An interaction between type VI and type IV collagens is likely to be physiologically significant, since type VI filaments and endothelial basement membranes are in close association in tissues. Therefore, this interaction was confirmed using isolated protein domains. The globular NC1 domain of type IV collagen was isolated from human amnion and purified by molecular sieve chromatography under native conditions (Fig. 1). The indicated fraction contained NC1, and the SDS-PAGE gel showed its purity and presence of both α1 and α2 chain dimers and monomers as previously reported (21).
For the interaction to be physiologically significant, the native amino-terminal globular domain of type VI collagen must have an affinity for the native carboxyl-terminal domain of type IV collagen. This was demonstrated by specifically immunoprecipitating a type VI-type IV complex from a bacterial collagenase digest of human amnion with either a polyclonal antibody specific to type IV collagen or a monoclonal antibody specific for ase digest of human amnion with either a polyclonal antibody have an affinity for the native carboxyl-terminal domain of type IV collagen and type VI collagen (Fig. 2). The protein was electrotransferred to a polyvinylidene difluoride membrane and the membrane was cut into three identical strips. Lanes 1 and 3 were developed with a guinea pig polyclonal antibody to the globular domain of α1(IV) and α2(IV), respectively, to show the positions of Dα, Dβ, and M (see Fig. 1). Lane 2 was treated with biotinylated type VI globular domains and developed with horseradish peroxidase and its substrate. The positions of the bands clearly show that the type VI globular domains only interact with the α1(IV) chain globular domain, since lanes 1 and 2 are identical and are different from lane 3. Arrowheads to the right mark positions of the molecular mass markers myosin (205 kDa), β-galactosidase (116 kDa), bovine serum albumin (80 kDa), and ovalbumin (49.5 kDa).

Fig. 2. Coimmunoprecipitation of type VI and type IV collagens. A, a partially purified bacterial collagenase digest of human amnion was treated with a polyclonal antibody to type IV collagen followed by protein G-Sepharose. The immunoprecipitate was separated in three identical lanes on SDS-PAGE using a 7.5% gel and then electrophoretically transferred to polyvinylidene difluoride. The membrane was developed as a Western blot with a polyclonal antibody to type IV collagen (lane 1), a preimmune serum (lane 2), and type VI collagen polyclonal antibodies (lane 3). Clearly, the immunoprecipitate contained both type IV and VI collagens. Arrowheads to the left identify type IV collagen-related bands, and arrowheads to the right show type VI collagen-related bands. B, a mixture of purified type VI and type IV globular domains were immunoprecipitated with a type VI collagen α3 chain amino-terminal domain-specific monoclonal antibody and analyzed as in A but using a 10% gel. The membrane was developed with a preimmune serum (lane 1), a polyclonal antibody against the type IV collagen NC1 domain (lane 2), and a type VI collagen α3 chain-specific monoclonal antibody (lane 3). Again the immunoprecipitate contained both type VI and type IV globular domains.

Fig. 3. Affinity blots of type IV collagen with biotinylated type VI collagen. A preparation of type IV collagen globular domains was separated on an 8–12% SDS-PAGE gradient gel in nonreducing conditions. The protein was electrophoretically transferred to a polyvinylidene difluoride membrane, and the membrane was cut into three identical strips. Lanes 1 and 3 were developed with a guinea pig polyclonal antibody to the globular domain of α1(IV) and α2(IV), respectively, to show the position of Dα, Dβ, and M (see Fig. 1). Lane 2 was treated with biotinylated type VI globular domains and developed with horseradish peroxidase and its substrate. The positions of the bands clearly show that the type VI globular domains only interact with the α1(IV) chain globular domain, since lanes 1 and 2 are identical and are different from lane 3. Arrowheads to the right mark positions of the molecular mass markers myosin (205 kDa), β-galactosidase (116 kDa), bovine serum albumin (80 kDa), and ovalbumin (49.5 kDa).
is 31% amino acid sequence identity between the two type VI amino-terminal domains.

Since interactions of type VI collagen with types I and II collagens have been reported, comparative solid phase binding studies were undertaken to determine the relative affinities of native type VI tetromers for various substrates. Binding to the NC1 domain of type IV collagen was found to be the strongest and was therefore fixed at 100% to normalize the results. On this scale, fibronectin was approximately 75%, and type VI binding to itself was at 65% (Fig. 5). Type V and XI collagens were at the level of BSA that was used as a control for nonspecific binding. Type I and type II collagen were about 10% above background and therefore weak compared with type IV collagen, fibronectin, and type VI collagen interactions. Binding of NC1 to type VI collagen was concentration-dependent and saturatable as shown in Fig. 6, with half-maximal binding at 0.2–0.3 μg type VI collagen. Binding to BSA was low and concentration-dependent. Binding to type I collagen was low and concentration-dependent but did not appear to reach saturation under the conditions used, indicating a low affinity for type VI collagen.

Mutations in type VI collagen give rise to Bethlem myopathy, whose major clinical feature is weakening of the muscles (29, 30). Fig. 7 shows the general co-distribution of type VI and type IV in human muscle using double immunofluorescent labeling of a section with Texas Red and fluorescein isothiocyanate-labeled secondary antibodies. The endomysium around the muscle fibers is stained, and the two patterns are identical. The immuno electron micrograph in Fig. 8 shows the close juxtaposition of gold-labeled type VI collagen filaments to the basement membrane. The filaments appear to form a continuous network connecting the basement membrane with the interstitial collagen fiber network.

**DISCUSSION**

The yeast two-hybrid system has not previously been used to screen for interactions of extracellular matrix connective tissue protein domains. It has, however, been used successfully to study the interactions of the extracellular domains of transmembrane proteins (91, 32). Numerous questions have been raised as to whether two-hybrid systems are applicable to the analysis of interactions between extracellular matrix proteins. Concerns include questions about proper folding of proteins, disulfide bond formation, targeting of extracellular matrix components outside the nucleus, and the absence of important post-translational modifications. These concerns can be appropriately addressed by first recognizing that, although the yeast two-hybrid system is unlikely to be universally applicable to the analysis of extracellular matrix interactions, it is a valid and useful tool for some applications as demonstrated here. It is unknown whether the protein-protein interaction actually occurs inside or outside of the nucleus (33). Consequently, it is possible that the initial interaction does not take place in the nuclear environment, and yeast have been shown to be quite favorable to folding and disulfide bond formation in recombinant proteins and domains (34). However, although secondary and tertiary structure frequently contribute to protein recognition and subsequent interaction, the basis for protein-protein interaction can also be embedded in the linear amino acid sequence of the interacting domains. This is critical to the applicability and success of many well accepted in vitro experimental procedures including binding studies using recombinant proteins or synthetic peptides, peptide libraries, Western blot analyses, and production of peptide antibodies that recognize native proteins. Judicious selection of bait domains maximizes the potential for success. This includes using protein domains that can fold independently, avoiding sequences that would trigger the protein outside the nucleus or that contain multiple cysteine residues. The use of libraries with small random primed inserts that are most likely to code single domains of matrix proteins is probably beneficial. It is also important to note that the time required to detect expression of β-galactosidase activity for extracellular matrix protein-protein interactions is usually longer than 12 h, which reflects the
lower affinities of these proteins (35) compared with many intracellular protein-protein interactions (33). However, reaction times should be less than 30 h, since false positives increase dramatically with longer times.

If the method were not applicable to extracellular matrix proteins, either no interactions or large numbers of nonspecific interactions would be detected. For the bait domains used here, a small number of positive clones were identified, which included two known extracellular matrix components. Furthermore, the selectivity of the interaction was demonstrated by showing that only the α1(VI) and not the α2(VI) amino-terminal domain interacted with α1(IV)-NC1 despite their high sequence similarity. Most importantly, the interaction was confirmed using isolated protein domains. The results of this study clearly demonstrate that two-hybrid analysis can be a powerful tool for identifying interacting domains of extracellular matrix structural proteins.

Our initial observations on the distribution of type VI collagen in skin led us to the conclusion that there was a much closer association with endothelial basement membranes around blood vessels and nerves than with the epithelial basement membranes (1). We speculated that perhaps endothelial basement membranes were anchored to the surrounding matrix by type VI collagen in much the same way that type VII anchoring filaments attach the epithelial basement membranes to the underlying dermis (36). More recent studies using immunogold electron microscopy co-localized type VI collagen and type IV collagen on the endothelial side of the glomerular basement membrane (37) and in basement membranes of the placenta (38). Here we show, by immunofluorescent labeling of muscle, a general co-localization of type VI with type IV collagen and a polyclonal antibody to type VI collagen produced the same result. Magnification: × 200.

Characterization of the interaction between type VI and type IV collagens identifies an important new physical link between basement membranes and the surrounding matrix. In a previous study, recombinant α(VI) chains did not bind to type IV collagen isolated from the EHS mouse tumor (8, 9). The discrepancy, however, is understandable given that the two-hybrid system is capable of detecting weak interactions that cannot be detected using solid phase binding assays (33, 39, 40). In addition, the strong binding to type IV collagen in the solid phase binding assays described here may be due to the presence of the globular domains of all three chains in a single native conformation in the type VI collagen used, as opposed to recombinant single domains used in other studies. Binding of intact type VI collagen to type I fibrillar collagen has been previously reported by Bonaldo et al. (2), and pepsin-solubilized type VI collagen to type II collagen has been reported by Badian et al. (4). However, a comparison of the relative affinities of the various collagens has not been reported previously. The major ligands for type VI collagen appear to be type IV
collagen, type VI collagen, and fibronectin. Although the interaction of type VI collagen is not been studied in detail, they have been co-localized in structures referred to as strings in developing avian cornea (together with type IV collagen) (41) and in plaques in mouse articular cartilage (42). They also interact in the detergent-insoluble pericellular matrix around fibroblasts (11, 12). The strong type VI/type VI interaction is probably important in the assembly and stability of beaded filaments in the extracellular space (17), so it is significant that the type VI/type IV interaction is even stronger. The interaction of type VI collagen with types I and II appears to be very weak with joint contractures and childhood onset. It is a heritable disorder, called Bethlem myopathy (29, 43). This is an F-actin, dystrophin, and syntrophins are intra-membrane interface.

Interaction of Type VI and Type IV Collagens

It appears that the interaction of type VI collagen with basement membranes involves multiple interaction sites, since the basement membrane proteoglycan perlecán also interacts with α2(VI) (8) and there may be other binding partners. It is therefore not possible to say exactly which interactions will be disturbed in Bethlem myopathy. If there are multiple binding sites on basement membranes for type VI collagen, this could also explain the mild phenotype, since the connection would not be completely broken, only weakened.

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Interaction of Type VI and Type IV Collagens

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