The NC1 Domain of Collagen IV Encodes a Novel Network Composed of the α1, α2, α5, and α6 Chains in Smooth Muscle Basement Membranes

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Type IV collagen, the major component of basement membranes (BMs), is a family of six homologous chains (α1–α6) that have a tissue-specific distribution. The chains assemble into supramolecular networks that differ in the chain composition. In this study, a novel network was identified and characterized in the smooth muscle BMs of aorta and bladder. The noncollagenous (NC1) hexamers solubilized by collagenase digestion were fractionated by affinity chromatography using monoclonal antibodies against the α5 and α6 NC1 domains and then characterized by two-dimensional gel electrophoresis and Western blotting. Both BMs were found to contain a novel α1α2α5α6 network besides the classical α1α2 network. The α1α2α5α6 network represents a new arrangement in which a protomer (triple-helical isoform) containing the α5 and α6 chains is linked through NC1-NC1 interactions to an adjoining protomer composed of the α1 and α2 chains. Re-association

The basement membrane (BM), a continuous sheet of extracellular matrix, separates epithelial cells from the underlying stroma and plays important roles in normal biological functions (such as cell adhesion, growth, and differentiation; tissue repair; and molecular ultrafiltration) as well as in pathological events (such as cancer cell invasion and metastasis). Moreover, degradation and de novo synthesis of vascular BMs are critical events in the angiogenesis processes. BMs function is impaired in hereditary and acquired diseases in which type IV collagen is affected, including Alport’s syndrome, a hereditary form of progressive renal disease; diffuse leiomymatosis, a benign proliferation of smooth muscle cells; and Goodpasture syndrome, an anti-type IV collagen autoimmune disease (1).

Type IV collagen is the major structural component of the BM, and it consists of a family of six homologous α(IV) chains, designated α1–α6 (1). Each chain is characterized by a long collagenous domain of ~1400 residues of Gly-X-Y repeats, interrupted by ~20 short noncollagenous sequences, and by a noncollagenous (NC1) domain of ~230 residues at the carboxyl terminus. Three α(IV) chains assemble into triple-helical molecules (protomers) that further associate to form supramolecular networks by dimerization at the carboxyl terminus through NC1 domains and by formation of tetramers at the amino terminus (2). The chain composition, and thus the properties of the type IV collagen networks are influenced by two factors. First, the chain composition of networks is limited by chain availability: the six chains show a tissue-specific expression pattern, with the α1 and α2 chains being ubiquitous and the α3–α6 chains having a more restricted distribution. Second, the NC1 domain confers specificity to the chain-specific assembly of networks (3); thus, unidentified recognition sequences must exist within the NC1 domain that direct the selection of chains to form triple-helical protomers and of triple-helical protomers to form networks. For instance, although the α1–α5 chains are coexpressed in glomerular BM, they segregate into an α1α2 network and a disulfide cross-linked α3α4α5 network, characterized by loops and supercoiling (4). The existence of the latter network provides an explanation for the glomerular BM defect in Alport’s syndrome, in which mutations in the genes encoding any of the α3, α4, and α5 chains perturb the assembly of the α3α4α5 network with the consequent loss of all three chains and progressive renal failure (3, 4).

Additional distinct type IV collagen networks must also exist in BMs of other tissues that contain the α5 and α6 chains along with the α1 and α2 chains, but are devoid of the α3 and α4 chains. These include the smooth muscle BMs of bladder, uterus, stomach, esophagus, small intestine, vasculature as well as the BMs of skin and the Bowman’s capsule of the kidney (5–8). The existence of a novel α5α6 network was suggested based on the immunohistochemical co-localization of these
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chains, but a link between α5 and α6 has not yet been biochemically proven. Mutations in the COLA45 gene encoding the α5 chain of type IV collagen also cause the loss of the α6 chain from these BMs in the X-linked form of Alport’s syndrome (9–11), presumably reflecting the co-assembly of the α5 and α6 chains into a single triple-helical monomer that self-assembles into an α5α6 network. In rare instances of X-linked Alport’s syndrome associated with diffuse leiomyomatosis, deletions occur in both the COLA45 gene and the adjacent COLA46 gene (encoding the α6 chain), leading to both renal failure and a benign proliferation of smooth muscle cells (12).

In this study, the network organization of the α5 and α6 chains of the smooth muscle BMs of aorta and bladder was investigated to acquire knowledge important for understanding their structure, function, and defects in disease. Surprisingly, the findings revealed the existence of a novel network composed of the α1, α2, α5, and α6 chains together, but not of the predicted network composed solely of the α5 and α6 chains. Evidence was obtained that supports our previous finding that the NC1 domains play a recognition role in the chain-specific assembly of networks. The linkage between the α5 and α6 chains in the α1α2α5α6 network provides a molecular explanation for the loss of both α5 and α6 chains in X-linked Alport’s syndrome, in which the α5 chain is mutated.

EXPERIMENTAL PROCEDURES

Proteins—Frozen bovine aorta, bladder, and kidney were purchased from Pel-Freez Biological (Rogers, AR) and stored at −20 °C. Aorta and bladder BMs were purified as previously described (13) and then digested with bacterial collagenase (Calbiochem), and the NC1 hexamers produced in rat using bovine glomerular BM hexamers as antigen (6).

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Truncated type IV collagen protomers were produced by digestion of BMs with pseudolysin (Pseudomonas aeruginosa elastase; EC 3.4.24.26; purchased from Nagase Biomedical, Fukushiyama, Japan) (14). Aorta BM (80 g of wet ground tissue) was digested with 0.5% Pseudomonas aeruginosa BMs with pseudolysin (E.C. 3.4.24.26) for 24 h at room temperature. The reaction products were separated according to their molecular mass by gel filtration on a Gel filtration followed by Western blot analysis with NC1-domain-specific mAbs. Recombinant human α1α6IV NC1 domains were expressed in human kidney 293 cells and purified as described previously (16).

Antibodies—For detection of α1–α5 NC1 domains of type IV collagen by Western blotting and immunohistochemistry, the H-series rat mAbs (H11, H22, H31, H43, and H52) were used (6). In addition, mAbs to the α5 NC1 domain (from Wieslab AB, Lund, Sweden) and a rabbit polyclonal antibody to the α6 NC1 domain, raised against an α6 NC1 synthetic peptide (17), were also used as indicated. The following precipitating mAbs were used for immunofinity purification of NC1 hexamers according to the chain composition. mAb1 to the α1 NC1 domain and mAb3 to the α5 NC1 domain (18–20) were purchased from Wieslab AB. Briefly, 5–10 μg of NC1 hexamers were incubated with mAb supernatant (0.1–0.5 ml) for 1 h at room temperature. The immune complexes were collected on protein G-Sepharose by gentle mixing with 30 μl of beads for 1 h at room temperature and then solubilized in sample buffer and analyzed by Western blotting. Precipitating mAbs were used for immunofinity purification of NC1 hexamers from the aorta and bladder BMs (0.2–0.5 mg) were applied to the mAb B51 and B66 affinity columns containing immobilized mAbs B51 and B66 were prepared by coupling protein G-purified mAb to Affi-Gel-10 columns (0.5–1 mg of IgG/0.5 ml of activated gel). For immunofinity purification, the NC1 hexamers from aorta or bladder BM (0.2–0.5 mg) were applied to the mAb B51 and B66 columns. The bound fraction was eluted with 0.5 M imidazole and then in the second dimension by SDS-PAGE using a 10–22% gel gradient. The gels were silver-stained or transferred to nitrocellulose filter paper for Western blot analysis. After blocking with 2% casein, the membranes were reacted with monoclonal antibodies to α1–α6 NC1 domains (diluted 1:500–1:1,000), incubated with the appropriate alkaline phosphatase-conjugated secondary antibody (diluted 1:2,000), and then developed with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.

In Vitro Hexamer Assembly—Dissociation and in vitro re-association of hexamers were performed as previously described (3). Native NC1 hexamers from aorta BM were dissolved by dilution (~0.5 μg/ml) into a solution of 50 mM formic acid buffered at pH 3.0 with Tris. Complete dissociation into NC1 monomers and dimers was verified by HPLC gel filtration using a Bio-Sil TSK 250 column (Bio-Rad). Reassembly of the α1–α6 NC1 subunits was performed by changing the buffer to Tris-buffered saline by repeated dilution-concentration cycles in Centricon-10 concentrators (Millipore Corp.) and then incubating the mixture (~1 mg/ml) for 24 h at room temperature. The reaction products were separated according to their molecular mass by gel filtration on a Superdex 200 fast protein liquid chromatography column (Amersham Pharmacia Biotech). The composition of the NC1 hexamers re-associated in vitro was analyzed as described for the native NC1 hexamers.

Rotary Shadowing Electron Microscopy—The protein samples (25 μg/ml) were diluted into a solution of 10 mM ammonium bicarbonate containing 60% glycerol, nebulized onto mica discs, evaporated in a Bal-Tec BA500K freeze-etch-replica system to 1.7 × 10−9 millitorrs, coated at an angle (rotation rate of 60 rpm) with 0.9 mM of platinum at 120 K, and then backed with 5 mM of carbon. The replicas were viewed with a JEM 100CXII electron microscope (Jeol Ltd., Tokyo, Japan) and photographed at a magnification of ×29,000.

RESULTS

Chain Composition of Type IV Collagen of Bovine Smooth Muscle BM

Previous studies have shown that the α6 and α6 chains, along with the α1 and α2 chains, are co-localized in the smooth muscle BMs of murine, canine, and human tissues (5–8). In this study, bovine aorta and bladder BMs were chosen as prototypes for the smooth muscle BMs of the vasculature and visceria because bovine tissues are amenable for isolation of sufficient quantities of type IV collagen for biochemical analyses (3, 4, 17, 23). Immunostaining with chain-specific mAbs (Fig. 1) showed that bovine aorta (a–g) and bladder (h–n) BMs contained the α1, α2, α5, and α6 chains, but were devoid of the α3 and α4 chains. The staining pattern for the aorta showed co-localization of the α1, α2, α5, and α6 chains in the smooth muscle BMs of the media. An arteriole, recognized as a circle of blue-colored nuclei and characterized by a thick wall of smooth muscle cells, also stained for all four chains in adventitia. In contrast, subendothelial BM, recognized as a thin layer of intima lining the upper end of the arterial wall, contained only the α1 and α2 chains. In bladder, the α1, α2, α5, and α6 chains were co-localized throughout the smooth muscle BMs of the lamina propria and media as well as in epithelial cell BM. The capillaries, recognized as rings with a thin membrane, contained only the α1 and α2 chains. These results show that the smooth muscle BMs of both bovine aorta and bladder are composed of α1, α2, α5, and α6 chains. These four chains can be arranged in several possible networks: separate α1–α2 and α5–α6 networks; a composite α1–α2α5–α6 network; or a mixture of networks with chain compositions of α1–α2α5, α1–α2α6, α1–α5α6, and α2–α5–α6. Because all chains were co-localized,
immunohistochemical analysis alone could not distinguish among these possibilities.

Arrangement of Chains in Protomers and Networks

Analysis of the Type IV Collagen Networks from Aorta BM by Digestion with Pseudolysin—Our previous studies (4, 23) showed that pseudolysin digestion solubilizes truncated protomers of collagen IV with retention of a portion of the triple-helical domain and the complete NC1-NC1 connection between protomers (Fig. 2a). Moreover, digestion of glomerular BM at 4 °C followed by digestion at 25 °C differentially solubilized two distinct networks, one composed of the α1 and α2 chains and the other composed of the α3, α4, and α5 chains (3, 4). Thus, pseudolysin cleavage can reveal information about the protomer and network organization of chains. In the case of aorta BM, rotary shadowing electron microscopy revealed that the truncated protomers existed as dimers, connected through NC1-NC1 domain interactions (Fig. 2b). Unlike glomerular BM, no differential solubilization of networks was observed for aorta BM, as the relative quantities of the NC1 domains of the α1, α2, α5, and α6 chains solubilized at 4 °C (Fig. 2c) and at 25 °C (Fig. 2d) did not differ. These results indicate that the α1, α2, α5, and α6 chains are arranged in triple-helical protomers, connected through NC1-NC1 interactions, forming one or more networks that are equally susceptible to pseudolysin digestion.

Precipitating mAbs to α5 and α6 NC1 Domains—The arrangement of the α1, α2, α5, and α6 chains was investigated by precipitation of the NC1 hexamers isolated from aorta and bladder BMs by collagenase digestion. The monomer and dimer subunits of an NC1 hexamer reflect the identity of the chains composing two triple-helical protomers that are adjointed by their NC1 domains forming a network (24, 25). In previous studies of glomerular BM, a strategy has been perfected to (a) separate NC1 hexamers of varying compositions using chain-specific mAbs that have the capacity to immunoprecipitate native hexamers and to (b) identify the chain identity of hexamer subunits by Western blot analysis using chain-specific monoclonal antibodies. In particular, mouse mAb1 and mAb3, targeted to the α1 and α3 NC1 domains, respectively, were used to separate two subpopulations of NC1 hexamers, an α1-α2 NC1 and an α3-α4-α5 NC1 hexamer, establishing the existence of the α1-α2(IV) and α3-α4-α5(IV) networks in glomerular BM (3, 18, 20).

In this study of the α5 and α6 chains, it was necessary to establish an additional set of mAbs that immunoprecipitate NC1 hexamers, but that are targeted to the α5 and α6 NC1 domains. In a previous study (6), mAbs B51 and B66 were produced using native NC1 hexamers from bovine glomerular BM as the immunogen. The specificity and precipitating properties of these mAbs were investigated herein. As shown in Fig. 3a, mAbs B51 and B66 are specific for the α5 and α6 NC1 domains, respectively, as determined by their reactivity against α1–α6 NC1 domains. The capacity of mAbs B51 and B66 to bind native NC1 hexamers was demonstrated by (a) their capacity to stain tissue sections without acid/urea treatment (data not shown), (b) their capacity to coprecipitate the α5 and α6 NC1 domains along with other NC1 domains in immu-
Fig. 3. mAbs B51 and B66 are novel precipitating mAbs for the α5(IV) and α6(IV) NC1 domains. a, the specificity of mAbs B51 (white bars) and B66 (black bars) was ascertained by ELISA against the six recombinant NC1 domains (a1–a6), coated at 100 ng/well in carbonate buffer, pH 9.5. b, antibody binding to native and dissociated NC1 hexamers was measured by ELISA. Renal BM hexamers were coated at 300 ng/well under native (phosphate-buffered saline; hatched bars) and dissociating (6 M guanidinium hydrochloride; black bars) conditions. mAbs B51 and B66 preferentially bound to native hexamers, similar to the precipitating mAb1 and mAb3. In contrast, non-precipitating antibody (non-pp. Ab) H52 and mAb5 raised against the α5 NC1 domain and the rabbit antibodies raised against the α6 NC1 domain, all produced against synthetic peptides, showed little reactivity for the native hexamers, but reacted well with the dissociated hexamers.

noprecipitation experiments or in affinity chromatography (see below), and (c) preferential reactivity with native rather than dissociated NC1 hexamers as measured by ELISA (Fig. 3b). Thus, mAbs B51 and B66 have the required properties for structural studies of NC1 hexamers, analogues to those of the well described mAb1 and mAb3 (see above). In contrast, the H-series of mAbs, previously prepared for the six distinct NC1 domains using short synthetic peptides rather than the NC1 hexamer (5), preferentially reacted with dissociated hexamer in ELISA (Fig. 3b) and were inactive toward native hexamers in the immunoprecipitation assay (data not shown), but they remain invaluable for Western blot analyses and tissue localization studies.

Fractionation and Composition of NC1 Hexamers from Aorta BM—The NC1 hexamers of aorta BM, chosen as a prototype for the vascular smooth muscle BMs, were solubilized by collagenase digestion and then fractionated according to their composition by immunoaffinity chromatography (or immuno precipitation) using mAbs B51 and B66 and mAb1. Collagenase digests the triple-helical collagenous domain,3 but leaves intact the NC1 hexamer that constitutes the interface between two adjacent protomers (Fig. 4, top). The unfractonated hexamers were characterized by Western blot analysis and shown to be composed of the α1, α2, α5, and α6 NC1 domains, but devoid of the α3 and α4 NC1 domains (Fig. 4a), consistent with the tissue staining patterns shown in Fig. 1a. The hexamers bound to mAb B51 contained all four NC1 domains, indicating that α5 coexists in hexamers with the α1, α2, and α6 NC1 domains (Fig. 4b); this fraction amounted to 12% of the total hexamers. Hexamers not bound to B51 mAb (the remaining 88%) contained exclusively α1 and α2 NC1 domains (Fig. 4c). The absence of the α6 NC1 domain in the unbound fraction demonstrates that the α6 NC1 domain always accompanies the α5 NC1 domain in hexamers, thus ruling out the existence of α6 homohexamers and of α1α6, α2α6, and α1α2α6 heterohexamers. Similarly, the hexamers bound to B66 mAb contained all four NC1 domains (Fig. 4d), whereas those not bound consisted of α1 and α2 NC1 domains only (Fig. 4e). This indicates that, in turn, the α5 NC1 domain always accompanies the α6 NC1 domain in hexamers, thus further excluding the existence of α5 homohexamers and of α1α5, α2α5, and α1α2α5 heterohexamers.

The allowable NC1 hexamer combinations that remain are the α1 and α2 homohexamers as well as the α1α2, α5α6, α1α5α6, α2α5α6, and α1α2α5α6 heterohexamers. To further distinguish among these possibilities, the aorta BM hexamers were fractionated with mAb1: all four NC1 domains were bound (Fig. 4f), and none remained unbound (Fig. 4g). This indicates that not only α2 NC1 domains, but also α5 and α6 NC1 domains must always be associated with α1 NC1 domains in hexamers, ruling out the existence of the α2 NC1 homohexamers as well as that of the α5α6 and α2α5α6

3 A small number of Gly-X-Y repeats (typically two) remain at the amino terminus of the NC1 domains solubilized by collagenase digestion of type IV collagen networks (35, 36). These short sequences constitute the junction between the triple-helical domain and the NC1 domain.

Fig. 4. Analysis of the NC1 hexamers from aorta BM. Top, native NC1 hexamers were solubilized from aorta BMs by collagenase digestion, purified, and analyzed by Western blotting (a) for the presence of α1–α6 NC1 domains. D and M indicate the positions of NC1 dimers and monomers on the Western blot, respectively. Center, the aorta NC1 hexamers were fractionated using mAbs B51 (b and c) and B66 (d and e) and mAb1 (f and g), and then the bound (b, d, and f) and unbound (c, e, and g) fractions were analyzed by Western blotting with chain-specific antibodies as described in the legend to Fig. 2. Bottom, the fractionation of the two NC1 hexamer populations of aorta BMs is shown schematically.
Western blotting with chain-specific antibodies as described in the
and B51 (respectively. Center, the bladder NC1 hexamers were fractionated using mAbs B51 (b and c) and B66 (d and e) and mAb1 (f and g), and then the bound (b, d, and f) and unbound (c, e, and g) fractions were analyzed by Western blotting with chain-specific antibodies as described in the
NC1 heterohexamers. The permitted combinations remaining
protomers that are adjoined through their respective NC1 do-
staining with chain-specific mAbs, as detailed below. Center, the
1 homodimers, a model of the α1α2 hexamer was pro-
2 and 1; α2 NC1 hexamers of bladder BM were separately
2 homodimers, three models for the α1α2α5α6 hexamer were proposed (j–l). Based on the identification of
hexamer were proposed (j–l). These results indicate that the basic
organization of NC1 hexamers is the same in the smooth muscle
BM of the bladder contains hexamers composed of α1α2 and
NC1 only, α1α2, α1α5α6, and α1α2α5α6 NC1. The first two represent the allowable combinations in the
hexamer fraction that did not bind to mAb B51 (or B66): the α1α2
NC1 hexamer must exist to account for the coprecipitation of α2 by mAb1, but the coexistence of an α1 homohexamer could not be ruled out because a precipitating mAb to α2 was not available. The last two represent the allowable combinations in the
hexamer fraction bound to mAbS B51 and B66. The α1α2α5α6 NC1 hexamer must exist to account for the coprecipitation of α2 by mAbs B51 and B66. The coexistence of the α1α5α6 NC1 hexamer cannot be entirely ruled out based on these data alone, but it is inconsistent with the two-dimen-
sional PAGE analysis (see below). Thus, aorta BMs consist of
two NC1 hexamer populations, α1α2 and α1α2α5α6, repre-
senting 88 and 12% abundance, respectively (Fig. 4, c–e), whose relationship to the parent hexamer is illustrated (b). Based on the identification of α1/α1 and α2α2 homodimers, a model of the α1α2 hexamer was pro-
posed (e). Bottom, the bladder α1α2α5α6 hexamers (~3 µg) were separated by two-dimensional PAGE, transferred onto nitrocellulose, and analyzed by Western blotting with mAbs H11 for α1 (c) and H22 for α2 (d). Based on the identification of α1/α5 and α2α6 heterodimers, three models for the
α1α2α5α6 hexamer were proposed (j–l).

Chain Organization of Adjoining Protomers—The two popu-
lations of NC1 hexamers, α1α2 and α1α2α5α6, reflect several possible combinations of chains that exist within triple-helical protomers that are adjoined through their respective NC1 do-
mains. The dimer subunits of these NC1 hexamers, observed under the dissociative conditions of SDS-PAGE, contain inter-
molecular disulfide bonds that connect NC1 domains of chains from each of two adjoining protomers (25–28). Thus, the iden-
tification of dimers with respect to their chains of origin estab-
lishes which chains are cross-linked by disulfide bonds and
therefore which chains exist in each of the two adjoining triple-
helical protomers (Fig. 6b). The identities of NC1 dimers as well as monomers were determined by two-dimensional gel electrophoresis followed by Western blot analysis with chain-specific mAbs (Fig. 6a). To simplify the analysis, the α1α2 and
α1α2α5α6 NC1 hexamers of bladder BM were separately studied.

The identities of monomers and dimers of the α1α2 NC1
hexamers of bladder BM were investigated first (Fig. 6, c–e). The monomers had several charge isoforms, whereas the
dimers had both charge and size isoforms, as previously

FIG. 5. Analysis of the NC1 hexamers from bladder BM. Top, native NC1 hexamers from bladder BMs were analyzed by Western blot
analysis (a) for the presence of α1–α6 NC1 domains. D and M indicate the positions of NC1 dimers and monomers on the Western blot, respec-
tively. Center, the bladder NC1 hexamers were fractionated using mAbs B51 (b and c) and B66 (d and e) and mAb1 (f and g), and then the bound (b, d, and f) and unbound (c, e, and g) fractions were analyzed by Western blotting with chain-specific antibodies as described in the
legend to Fig. 2. Bottom, the two NC1 hexamer populations thus iden-
tified in bladder BMs are schematically shown.

FIG. 6. Organization of the chains in the NC1 hexamers ana-
yzed by two-dimensional gel electrophoresis. Top, the NC1 hex-
amers from bladder BM (10 µg) were separated by two-dimensional PAGE, transferred to nitrocellulose, and stained for total protein with colloidal gold (a). Under these conditions, the NC1 hexamer was re-
solved into its subunits, NC1 monomers (M) and disulfide-linked dimers (D), whose relationship to the parent hexamer is illustrated (b). The assignment of individual protein spots was based on the pattern of staining with chain-specific mAbs, as detailed below. Center, the
bladder α1α2 hexamers (~2 µg) were separated by two-dimensional PAGE, transferred onto nitrocellulose, and analyzed by Western blotting with mAbs H11 for α1 (c) and H22 for α2 (d). Based on the identification of
α1/α1 and α2α2 homodimers, the bladder α1α2 hexamer was pro-
posed (e). Bottom, the bladder α1α2α5α6 hexamers (~3 µg) were separated by two-dimensional PAGE, transferred onto nitrocellulose; and analyzed by Western blotting with mAb H11 for α1 (f), mAb H22 for α2 (g), mAb5 for α5 (h), and mAb B66 for α6 (i). Based on the identifi-
cation of α1α5 and α2α6 heterodimers, three models for the
α1α2α5α6 hexamer were proposed (j–l).

Not Bound Bound ~30% ~70% a b c d e f g h i j k l α1α2α5α6 hexamers: α1α2 α1α5 α1α2α5 α2α6 α1α2α5α6 NC1 domains, representing 70 and 30% abundance, respectively (Fig. 5, bottom). These results indicate that the basic
organization of NC1 hexamers is the same in the smooth muscle
BM of two distinct tissues.
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![Diagram](http://www.jbc.org/)

**Fig. 7. Analysis of NC1 hexamers re-associated in vitro from dissociated bovine aorta BM hexamers.** HPLC gel filtration showed that the aorta BM hexamers (H) were completely dissociated into NC1 monomers (M) and dimers (D) at pH 3 (b). After dissociation, the NC1 hexamers were re-associated in vitro and separated by HPLC gel filtration (c). The composition of hexamers re-associated in vitro was analyzed by fractionation with mAb1 (d and e) and mAb B51 (f and g), followed by Western blot analysis of the bound (d and f) and unbound fractions (e and g) as described for the native hexamers of aorta BM in the legend to Fig. 4.

In Vitro Reassembly of NC1 Hexamers from Smooth Muscle BM—In a previous study, we found that the NC1 domains contain recognition sequences that are sufficient to encode the assembly of a1-a2 and a3-a4-a5 NC1 hexamers of glomerular BM (3). Likewise, it was of interest to determine whether the NC1 hexamers of aorta BM, after dissociation into monomers and dimers, had the capacity to reassemble into the two distinct a1-a2 and a1-a2-a5-a6 NC1 hexamers. This was investigated by comparing the composition of native hexamers from aorta BM with that of in vitro reconstituted hexamers using the experimental approach outlined in Fig. 7. Complete dissociation into NC1 monomers and dimers was achieved by dilution at pH 3.0, as shown by HPLC gel-filtration analysis (Fig. 7b). Under re-associative conditions, most (>95%) NC1 domains reassembled into hexamers, with only ~3% forming higher molecular mass aggregates (Fig. 7c). The composition analysis of the re-associated hexamers using mAb1 and B55 antibodies clearly showed that the re-associated hexamers were distributed in the same two subpopulations as the native hexamers. mAb1 antibodies quantitatively precipitated the a1, a2, a5, and a6 NC1 domains (Fig. 7d), with none remaining in the unbound fraction (Fig. 7e). In contrast, mAb B55 bound all a5 and a6 and some a1 and a2 NC1 domains (Fig. 7f), but some a1 and a2 NC1 domains remained unbound (Fig. 7g). These hexamer populations were identical to those found for native hexamers of aorta BM (above), indicating that the NC1 domains specify the assembly of distinct hexamers. Similar results were obtained upon re-association of purified fractions containing NC1 monomers only or NC1 dimers only (data not presented).
FIG. 8. Triple-helical isoforms of type IV collagen and their interactions through the NC1 domains. Three kinds of NC1 hexamers with distinct compositions have been identified. The α1α2 hexamer (a) was first demonstrated chemically by studies with type IV collagen from the Engelbreth-Holm-Swarm tumor or human placenta and was second demonstrated by immunoprecipitation with mAb1 (to the α1 NC1 domain) of hexamers from glomerular BM (3, 20). The α3α4α5 hexamer (b) was identified by immunoprecipitation with mAb3 (to the α3 NC1 domain) of hexamers from glomerular BM (3, 20). The existence of an α1α2α5α6 hexamer (c) was established in this study by immunoprecipitation with mAb1 and mAbs B51 and B66. These hexamers are derived from three basic kinds of triple-helical isoforms that differ in composition and stoichiometry of the six chains of type IV collagen: (α1)zα2, α3α4α5, and (α5)zα6.  

shown), indicating that the reassembly of an α1α2α6α6 hexamer in vitro can proceed even in the absence of NC1 dimers, as previously found for the reassembly of α1α2 and α3α4α5 NC1 hexamers in vitro (3).

**DISCUSSION**

Random combinations of the six chains (α1–α6) of type IV collagen allow for 56 different protomers (triple-helical isoforms), which can further self-associate, forming a multiplicity of networks that differ with respect to which isoforms are connected through NC1-NC1 interactions (3). Studies to date indicate that only a few of these arrangements exist in BMs. All mammalian BMs appear to contain an α1α2 network, assembled from (α1,α2) protomers that interact with each other at the carboxyl termini through their NC1 domains (Fig. 5a) and at the amino terminus through their 7 S region. Certain BMs contain additional specialized networks such as the α3α4α5 network of renal glomerular BM (3, 4), which self-assembles similarly from α3α4α5 protomers (Fig. 8b). This study was designed to elucidate the organization of the remaining α6 chain, which is known to coexist with the α5 chain in smooth muscle BMs. Aorta and bladder BMs were chosen for study, as they could be isolated in sufficient quantities for biochemical analyses. The experimental strategy required the establishment of two new mAbs directed against the α5 and α6 NC1 domains (mAbs B51 and B66) for use in the immunoaffinity fractionation of NC1 hexamers.

In the smooth muscle BMs of both aorta and bladder, the α6 chain was found to assemble with the α5 chain, forming a novel (α5)zα6 triple-helical protomer, which further interacts with an (α1,α2) protomer through the NC1 domains to yield an α1α2α5α6 hexamer (Fig. 8c). The “classical” network composed of the α1 and α2 chains was found to coexist with the α1α2α5α6 network in smooth muscle BMs, amounting to 70% in aorta BM and 82% in bladder BM. The α1α2 network also composed the subendothelial BM of the aorta, which is devoid of the α1α2α5α6 network (Fig. 9), as well as the corresponding BM of capillaries in the bladder.

We recently obtained evidence that the recognition mechanism governing the chain-specific assembly of the α1α2 and α3α4α5 networks of glomerular BM is encoded in the NC1 domains (3). Herein, a similar study was conducted with a mixture of α1α2 and α1α2α5α6 hexamers from smooth muscle BM. The native hexamers were dissociated into NC1 monomers and dimers, which were then reassembled in vitro. The organization of reconstituted hexamers duplicated that of native hexamers, demonstrating the specificity of interactions among NC1 domains. Thus, the NC1 domains of smooth muscle BM encode the chain-specific assembly of the α1α2 and α1α2α5α6 networks, as illustrated in Fig. 10. Additionally, the recognition mechanism must allow for the α5 chain to assemble into two different networks: the α3α4α5 and α1α2α5α6 networks. This may explain why, in various tissues, the expression of the α3 and α4 chains (in glomerular BM) is segregated from the expression of the α6 chain (in smooth muscle, skin, and Bowman’s capsule BMs). Overall, these studies of glomerular and smooth muscle BMs indicate that the NC1 domains play a fundamental recognition function in the chain-specific assembly of networks: first, by specifying the selection of chains for protomer assembly, and second, by specifying the selection of protomers for network assembly.

Two distinct types of recognition sequences must specify the assembly of triple-helical isoforms and the interaction of protomers through NC1 domains, respectively. In the assembly of the α1α2 and α3α4α5 networks of glomerular BM, both types of interaction are specific, resulting in distinct hexamers and networks. In smooth muscle BM, specificity is manifested in the assembly of distinct (α1)zα2 and (α5)zα6 protomers; but subsequently, these protomers interact with each other to form a mixed network. This is presumably the result of higher homology between α1 and α5 NC1 domains in the α1-like subfamily (∼83% sequence identity compared with ∼69% for α1–α3 and ∼68% for α3–α5). We conjecture that residues divergent between the α1 and α5 NC1 domains (∼17%) encode the specific assembly of distinct protomers in the chain selection step, whereas residues identical in both the α1 and α5 NC1 domains but different in α3 (∼18%) encode the protomer selection step. Thus, the (α1,α2) protomers may interact with each other or with the more homologous (α5)zα6 protomers, but not with the α3α4α5 protomers. A similar reasoning would apply to the α2-like subfamily of NC1 domains, with the α2–α6 pair having more homology (∼76% sequence identity) than the α2–α4 (∼69%) and α4–α6 (∼67%) pairs.

D.-B. Borza, O. Bondar, P. Todd, and B. G. Hudson, manuscript in preparation.
The existence of an α1α2α5α6 network provides a molecular explanation for the loss of the α6 chain from BMs when the α5 chain is mutated in X-linked Alport’s syndrome. It is well established that both the α5 and α6 chains are absent in the kidneys and skin of human patients with mutations in the COL4A5 gene and in multiple tissues (including skin, kidney, bladder, and lung) of a canine model of Alport’s syndrome characterized by a COL4A5 nonsense mutation (7). Thus, the loss of the α6 chain is a consequence of mutation of the α5 chain, which causes failure of assembly of the α5α6 protomer and of the α1α2α5α6 network and/or degradation of the mutated network. This mechanism is analogous to that for the loss of the α3α4α5 network from glomerular BM when the α5 chain is mutated (3,4). The loss of the α3α4α5 network leads to deterioration of glomerular BM and progressive renal failure over 5–20 years, whereas the loss of the α1α2α5α6 network from smooth muscle tissues does not lead to apparent pathology, raising the issue of the biological significance of the latter network (7). The α3α4α5 network is required for long-term stability of glomerular BM and glomerular filtration function, possibly conveying resistance to the action of proteolytic enzymes (31,32). The long-term consequences of the loss of the α1α2α5α6 network on the function of smooth muscle in various tissues, if any, have not yet been reported. The α6 chain is postulated to be critical for smooth muscle function based on the pathology associated with mutations in the COL4A6 gene. Mutations in both the COL4A5 and COL4A6 genes occur in patients with Alport’s syndrome associated with diffuse leiomyomatosis (12,33). The combined mutations are associated not only with progressive renal failure, as seen in Alport’s syndrome patients, but also with smooth muscle cell proliferation, forming benign tumors of the esophagus, tracheobronchial tree, and genital tract. However, recent studies with the canine model of Alport’s syndrome have shown that smooth muscle cell proliferation is not a characteristic of pure Alport’s syndrome, even though both the α5 and α6 chains are absent in the corresponding smooth muscle tissues of the canine model (7). Moreover, both the α5 and α6 chains are predicted to be absent in the smooth muscle BMs of human patients with Alport’s syndrome, whether associated or not with diffuse leiomyomatosis, because of the existence of the α1α2α5α6 network. Thus, the absence of this network is not sufficient to cause diffuse leiomyomatosis; therefore, pathology of diffuse leiomyomatosis does not underscore the functional importance of the α6 chain. It is likely that diffuse leiomyomatosis is a result of mutations in a yet undiscovered gene near the locus of the COL4A6 gene, as postulated by others (34).

In conclusion, the present findings, along with previous ones, indicate that the six chains of type IV collagen are distributed in three supramolecular networks whose chain compositions are encoded by the NC1 domains. The six chains assemble into three major triple-helical protomers with molecular compositions of (α1)2α2, (α3α4α5), and (α5)2α6. These protomers assemble into three major networks (α1α2, α3α4α5, and α1α2α5α6), in which they are connected by NC1-NC1 interactions. The α1α2 network exists in all mammalian BMs, whereas the α3α4α5 and α1α2α5α6 networks are restricted to certain BMs, such as glomerular and smooth muscle BMs, respectively. Presumably, the tissue-specific distribution of networks confers specialized functions to various BMs.

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The NC1 Domain of Collagen IV Encodes a Novel Network Composed of the α1, α2, α5, and α6 Chains in Smooth Muscle Basement Membranes
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