The Structural Organization of Type IV Collagen

IDENTIFICATION OF THREE NC1 POPULATIONS IN THE GLOMERULAR BASEMENT MEMBRANE*

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Type IV collagen, which has long been assumed to contain two α1(IV) and one α2(IV) chains, also contains α3(IV), α4(IV), and α5(IV) chains. Stoichiometry of collagenous α(IV) chains differs among tissues, suggesting the existence of subclasses of type IV collagen, each with a unique chain composition. This study seeks to define, by characterization of subunit compositions of NC1 domain populations, the structural organization of type IV collagen from bovine glomerular basement membrane.

NC1 hexamers from type IV collagen were separated on two affinity chromatography columns, one containing monoclonal antibodies to the α3 chain, and another, to the α1 chain. SDS-polyacrylamide gel electrophoresis, immunoblotting, reversed phase high-performance liquid chromatography, and enzyme-linked immunosorbent assay identified three NC1 hexamer populations: 1) a hexamer composed of (α1)3 and (α2)3 homodimers; 2) a hexamer composed of (α3)2 and (α4)2 homodimers; 3) a hexamer containing all four α chains connected in heterodimers, α1−α3 and α2−α4. Results suggest that there are two distinct type IV collagen molecules, one composed of α1(IV) and α2(IV) chains and another composed of α3(IV) and α4(IV) chains. Furthermore, polymerization occurs between molecules with the same chain composition and between molecules with different chain composition. Moreover, crosslinking between different α chains is restricted, thus limiting the number of possible macromolecular structures.

Type IV collagen, with its self-aggregating properties, forms a network comprising the backbone of basement membranes, and is thus one of their major structural components. Each type IV collagen molecule is composed of three α chains that can be divided into three domains: 7 S, a minor collagenous domain in the N-terminal region; a major collagenous domain in the middle region; and NC1, a noncollagenous globular domain in the C-terminal region. The trimers of α chains are organized into triple helices in the 7 S and the major collagenous domains, but in the NC1 domain each chain is folded into a globular structure, stabilized by intrachain disulfide bonds. The 7 S and NC1 domains are cross-linking domains. Four type IV collagen molecules are connected in the 7 S region, and two molecules in the NC1 domain, giving rise to a network of collagen IV molecules.

Bacterial collagenase digestion of basement membranes yields hexameric forms of the NC1 domains derived from two type IV collagen molecules connected in this region. These NC1 hexamers can be dissociated with denaturing agents to monomeric (M) and dimeric (D) subunits originating from cross-linking of the α chains (2). The classical type IV molecule is composed of α1 and α2 chains. However, the recent localization of Goodpasture antigen revealed two novel α(IV) chains, α3(IV) and α4(IV) (3), and the gene for a fifth chain, α5(IV), has been identified (4).

These five chains can be combined into 35 different trimers or subtypes, each with a unique chain composition. The trimers can be connected in many arrangements and differ in distribution in basement membranes of various tissues. It has been shown that α3(IV) and α4(IV) chains co-localize and are only present in basement membranes of the kidney, eye, cochlea, lung, and brain, whereas the α1(IV) and α2(IV) chains are present in all basement membranes (5). Basement membranes of the glomerulus, placenta, and lens capsule differ with respect to the number of α3(IV) and α4(IV) chains (6), and α3(IV) and α4(IV) chains have limited distributions in different basement membranes within the kidney (7). These findings suggest that there exist at least two different types of collagen IV molecules, one composed of classical chains, α1(IV) and α2(IV), and a second containing the α3(IV) and α4(IV) chains.

To test that hypothesis, NC1 hexamers from bovine glomerular basement membranes (GBM)† were applied to two affinity chromatography columns, the first with monoclonal antibodies to α1(IV)NC1 and the second with monoclonal antibodies to α3(IV)NC1. This "two-step" method isolated three distinct NC1 hexamer populations: one composed of α1(IV)NC1 and α2(IV)NC1 chains, a second composed of α3(IV)NC1 and α4(IV)NC1, and a third with a mixed composition of all four α(IV)NC1 subunits.

MATERIALS AND METHODS

Preparation of NC1 Hexamers—Basement membrane was prepared from bovine kidneys as described previously (8). Bacterial collagenase (Worthington) digestion was performed in 50 mM HEPES buffer, pH 7.5, with 10 mM CaCl2 and the following protease inhibitors: 5 mM PMSF, 50 mM sodium fluoride, 5 mM EDTA, 1 mM EGTA, 100 mM dithiothreitol, 50 mM hydroxylamine, 100 mM benzamidine, 100 mM phenylmethanesulfonyl fluoride, 100 μM pepstatin, 100 μM aprotinin, and 100 μM leupeptin. The basement membranes were dissolved in 0.5 M sodium chloride, 0.1% Na2EDTA, 0.1% sodium deoxycholate, and 1% sodium dodecyl sulfate at pH 8.5, and the digestion was allowed to proceed for 16–24 h at 37°C. The digestion was terminated by the addition of 300 mM NaCl, and the samples were clarified by centrifugation.

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benzamidine, 25 mM ε-aminocaproic acid, 4 mM N-ethylmaleimide, 1 mM phenylmethylsulfonyl fluoride using 10 mg/ml GBM and 0.01 mg/ml of collagenase. Digestion was performed under continuous stirring at 37°C for 20 h, and the solubilized basement membrane was then ultracentrifuged at 100,000 × g for 1 h. The supernatant was applied against 2 × 20 volumes of Tris-HCl, 0.15 M NaCl, 0.02% (w/v) NaN₃, pH 7.5, and applied to a DE52-Sepharose ion exchange chromatography column (Pharmacia, Uppsala, Sweden) equilibrated in the same buffer. NC1, which does not bind to the column under these conditions, was concentrated in an ultrafiltration cell (YM10 Amicon) and applied to an S-200 gel filtration column (Pharmacia) equilibrated with 50 mM Tris-HCl, 0.15 M NaCl, 0.02% (w/v) NaN₃, pH 7.5. Fractions from the main peak, containing the NC1 hexamer, were pooled and used for subsequent studies.

**Antibodies**—Two monoclonal antibodies (mAb), mAb 6 and 17 (9), specific for α1(IV)NC1 and α3(IV)NC1, respectively, were used for the preparation of two affinity chromatography columns and in ELISA and immunoblot analysis. Additionally two polyclonal antibodies (pAb), pAb 2 and 4 (7), restricted to α2(IV)NC1 and α4(IV)NC1, respectively, were used in ELISA and immunoblot analysis.

**Chromatography**—Two affinity chromatography columns were prepared using 2 ml of activated agarose gel (MemiLeak, BioCarb AB, Lund, Sweden) to which 1.8 mg of mAb 6 and 2 mg of mAb 17, respectively, were coupled by the procedure described in the product insert. The columns were equilibrated with 50 mM Tris-HCl, 0.15 M NaCl, 0.02% (w/v) NaN₃, pH 7.5, and the samples were applied in the same buffer. Bound material was eluted with 50 mM sodium citrate, 6 mM urea, 0.02% (w/v) NaN₃, pH 4.0. The pH of the eluted protein was immediately adjusted to 7.0, and it was dialyzed against 3 × 100 volumes of starting buffer. Protein concentrations were estimated by absorbance at 280 nm, and amounts of separated NC1 hexamer subclasses were calculated from the chromatograms.

Material separated by affinity chromatography was further analyzed by reversed phase HPLC. Samples were acidified by adding 25% (v/v) trifluoroacetic acid and then applied to a C4 column (4.6 mm × 15 cm) (Vydac, Hesperia, CA) equilibrated with 90% acetonitrile, 0.1% trifluoroacetic acid. Bound material was eluted with a linear gradient from 31% to 42% acetonitrile, 0.1% trifluoroacetic acid in 33 min at 1.2 ml/min. Fractions were collected and analyzed for α1(IV)NC1 and α3(IV)NC1 by ELISA.

**Electrophoresis and Immunoblotting**—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done as described by Laemmli (10), in 10–16% gradient gels. The gels were stained with silver according to the method of Morrissey (11). Immunoblot experiments were performed on samples that were separated with SDS-PAGE and transferred to nitrocellulose paper as described by Burnett (12) using a semi-dry electroblotter. Transfers were performed at 20 V for 1 h with a semi-dry transfer apparatus (DAKO A/S, Glostrup, Denmark). Nonspecific binding sites on the nitrocellulose paper were blocked with 2% BSA in 50 mM sodium carbonate, 0.05% (v/v) Tween 20. 100 μl of primary antibodies (mAb 6, mAb 17, pAb 2, or pAb 4, diluted in phosphate-buffered saline containing 0.2% bovine serum albumin, pH 7.5 (BSA-PBS) (1.5 mM KH₂PO₄, 8 mM Na₂HPO₄, 0.12 m NaCl, 2.5 mM KC, 0.05% (v/v) NaN₃). They were then washed 3 times with 0.15 M NaCl, 0.05% (v/v) Tween 20, and incubated for 1 h with alkaline phosphatase-conjugated rabbit anti-mouse Ig (DAKO A/S) diluted 1:1000 in BSA-PBS or swine anti-rabbit IgG (Orion Diagnostics AB, Trosa, Sweden) 250-fold diluted in BSA-PBS. The papers were then washed 3 times, and the bound alkaline phosphatase activity was visualized using bromochloroindolyl phosphate/nitro blue tetrazolium substrate (13).

**Enzyme-linked Immunosorbent Assay (ELISA)**—Polystyrene microtiter plates (Nunc Immunoplate, Roskilde, Denmark) were coated with affinity chromatography fractions and fractions collected from the reversed phase HPLC separation, dilute 10 times in 50 mM Tris-HCl, 6 µM guanidine-HCl, pH 7.4. The plates were incubated overnight at room temperature and then washed three times with 0.16 M NaCl, 0.006 M Tween 20. 100 μl of primary antibodies (mAb 6, mAb 17, pAb 2, and pAb 4) diluted in BSA-PBS, pH 7.3, were added to each well. After incubation at room temperature for one hour the plates were washed three times as above. Alkaline phosphatase-conjugated rabbit anti-mouse Ig (DAKO A/S) diluted 1:1000 or swine anti-rabbit IgG (Orion Diagnostics AB) diluted 1:200 in BSA-PBS were added and allowed to incubate for 1 h at room temperature. This was followed by 3 washes and then alkaline phosphatase substrate, p-nitrophenyl phosphate (Sigma), at 1 mg/ml in 1 M diethanolamine, 0.5 mM MgCl₂, pH 9.8, was added in 100-μl aliquots to each well. Color development was measured spectrophotometrically at 405 nm.

**RESULTS**

We have previously shown that NC1 domains solubilized from type IV collagen by collagenase digestion and further purified by ion-exchange chromatography and gel filtration exist as hexamers originating from two type IV collagen molecules connected in this region (14).

When NC1 hexamers were applied to an anti-α3(IV)NC1 affinity column (Fig. 1) about 25% of the hexamers bound to the column (Fig. 2a). SDS-PAGE analysis showed that the bound hexamers contained all four monomer bands, whereas the unbound hexamers contained two of the monomer bands present in the bound fraction and one new band, with a lower molecular weight (Fig. 2b). Immunoblot analysis showed that the two fractions had different α(IV) chain compositions. Hexamers that bound to the column contained all four α(IV) chains, α1(IV), α2(IV), α3(IV), and α4(IV), while unbound hexamers contained only α1(IV) and α2(IV) chains (Fig. 2c).

The unbound fraction was then applied to an anti-α1(IV) affinity column to find out if it contained only one hexamer population, composed of α1(IV) and α2(IV) chains (Fig. 3). The bound and unbound fractions were analyzed as described above. Most of the material bound to the column (Fig. 3a) and gave the same SDS-PAGE pattern as before application to the column, except for the low molecular weight band found in the unbound material (Fig. 3b). That band had a lower molecular weight than any of the known α(IV)NC1 monomers. Immunoblot analysis of the bound hexamers showed that they contained both α1(IV)NC1 and α2(IV)NC1 chains, and these chains were cross-linked into homodimers. Staining with specific antibodies showed that the dimer bands of α1(IV)NC1 and α2(IV)NC1 did not migrate to the same position as those of the monomers. This suggests that α1(IV)NC1 and α2(IV)NC1 are structurally different from α1(IV)NC1 and α2(IV)NC1.

**FIG. 1. Scheme for resolution of type IV collagen NC1 hexamer populations.** NC1 hexamers from bovine GBM were applied to an anti-α3(IV)NC1 affinity column, and the bound and unbound fractions were applied separately to an anti-α1(IV)NC1 affinity column. Bound and unbound fractions from both anti-α3(IV)NC1 and anti-α1(IV)NC1 affinity columns were analyzed by SDS-PAGE, immunoblotting, reversed-phase HPLC, and ELISA. Material that did not bind to any column was analyzed by SDS-PAGE and immunoblotting.
**FIG. 2.** Affinity chromatography of NC1 on an anti-α3(IV)NC1 column and analysis of products. NC1 subunits contained in unbound pool I and bound pool II from the affinity column (panel a) were resolved by SDS-PAGE and stained with silver (panel b). Dimers (D), monomers (M), and positional markers for the four monomer subunits are shown in panel b. Immunoblot analysis of pools I and II with antibodies specific for α1(IV)NC1, α2(IV)NC1, α3(IV)NC1, and α4(IV)NC1 revealed that pool I contained α3(IV)NC1 and α2(IV)NC1, whereas pool II contained all four α(IV)NC1 subunits (panel c). NC1 was applied to the column in 0.05 M Tris-HCl, 0.15 M NaCl, 0.02% (w/v) NaN₃, pH 7.5 (buffer A), and bound proteins were eluted with 0.05 M sodium citrate, 6 M urea, 0.02% NaN₃, pH 4.0 (buffer B).

**FIG. 3.** Affinity chromatography of unbound NC1 pool I (Fig. 2a) on an anti-α1(IV)NC1 column. Unbound pool I of Fig. 2a, containing proteins that did not bind the anti-α3(IV)NC1 affinity column, was applied to an anti-α1(IV)NC1 column. Unbound (panel a, pool I) and bound (panel a, pool II) fractions were collected. The contents of pool I and II were resolved by SDS-PAGE and stained with silver (panel b). Dimers (D), monomers (M), and the unknown monomer band in pool I (arrow) are indicated. Proteins in pools I and II were analyzed by immunoblotting with antibodies to the NC1 domain of α1(IV), α2(IV), α3(IV), and α4(IV) chains (panel c). The unbound material (pool I) did not contain any α chains, whereas the bound material contained α1(IV)NC1 and α2(IV)NC1. Antibodies to α1(IV)NC1 and α2(IV)NC1 stained different dimer bands, indicating these dimeric subunits are homodimers (α1(IV)NC1)₂ (large arrows) and (α2(IV)NC1)₂ (small arrows).

position; thus there are no heterodimers consisting of α1(IV)NC1 and α2(IV)NC1. Immunoblot analysis showed that the unbound material did not react with any of the antibodies to α1(IV)NC1, α2(IV)NC1, α3(IV)NC1, or α4(IV)NC1 (Fig. 3c). Therefore, the hexamers that passed through the anti-α3(IV)NC1 affinity column, but bound to the anti-α1(IV)NC1 affinity column, are composed of monomers and homodimers of α1(IV)NC1 and α2(IV)NC1 chains. A hexamer composed of only α1(IV)NC1 chains might be present, but not a hexamer of only α2(IV)NC1 chains, because that kind of hexamer would not bind to the anti-α1(IV)NC1 affinity column. The low molecular weight band found in the unbound fraction could not be identified and may have been composed of impurities from the hexamer preparation.

When hexamers that bound to the anti-α3(IV)NC1 affinity column, containing all four α(IV)NC1 chains, were applied to the anti-α1(IV)NC1 affinity column (Fig. 4), about 60% bound to the column (Fig. 4a). SDS-PAGE analysis of the bound
and unbound material showed that the bound hexamers contained all four monomer bands, while the unbound hexamers contained only two high molecular weight monomer bands, the α3(IV) and α4(IV) (Fig. 4b). ELISA (not shown) and immunoblot analysis confirmed that bound hexamers contained all four α(IV) chains (Fig. 4c). Anti-α1(IV) and anti-α3(IV) antibodies stained bands that had migrated to the same dimer positions, suggesting cross-linked heterodimers of α1(IV)NC1 and α3(IV)NC1. Anti-α2(IV) and anti-α4(IV) also showed the same reactivity suggesting heterodimers of α2(IV)NC1 and α4(IV)NC1. Thus, this hexamer population is composed of all four α(IV) chains connected in two distinct heterodimers, α1(IV)α3(IV) and α2(IV)α4(IV). The bound material might contain a hexamer composed of only α1(IV)α3(IV) dimers but not a hexamer composed of only α2(IV)α4(IV) dimers, since such hexamers would not bind to an anti-α3(IV) or an anti-α1(IV) affinity column. Immunoblot analysis of the unbound material showed staining only of the α3(IV) and α4(IV) chains, with no sharing of position by the dimers (Fig. 4c). This pattern shows that there also is a hexamer population composed of only α3(IV)NC1 chains, but not of only α4(IV)NC1 chains, since such a hexamer would not bind to the anti-α3(IV)NC1 affinity column.

In summary, three hexamer populations were separated using an affinity column with antibodies to α3(IV)NC1 followed by another column with antibodies to α1(IV)NC1 (Fig. 5): 1) a hexamer containing α1(IV)NC12 and α2(IV)NC12 homodimers, 2) a hexamer containing α3(IV)NC12 and α4(IV)NC12 homodimers, and 3) a hexamer containing α1(IV)NC1-α3(IV)NC1 and α2(IV)NC1-α4(IV)NC1 heterodimers. The same results were obtained when the affinity chromatography columns were used in the opposite order.

**FIG. 4.** Affinity chromatography of bound NC1 pool II (Fig. 2a) on an anti-α1(IV)NC1 column. Bound pool II of Fig. 2a, containing proteins that bound to the anti-α3(IV)NC1 affinity column, was applied to an anti-α1(IV)NC1 affinity column (panel a). Unbound (pool I) and bound (pool II) fractions were collected. Proteins in pool I and II were separated by SDS-PAGE, stained with silver (panel b), and immunoblotted with antibodies to the NC1 domains of α1, α2, α3, and α4 chains of type IV collagen (panel c). Unbound pool I contained only α3(IV)NC1 and α4(IV)NC1, whereas bound pool II contained all four α1(IV)NC1 through α4(IV)NC1 subunits. Antibodies to α3(IV)NC1 and α4(IV)NC1 subunits stained different dimer bands in the unbound pool I, indicating homodimers α3(IV)NC12 (large arrows) and α4(IV)NC12 (small arrows). Antibodies to α1(IV)NC1 and α3(IV)NC1 bind to the same dimer bands in bound pool II, suggesting heterodimers of α1(IV)NC1 and α3(IV)NC1; α2(IV)NC1 and α4(IV)NC1 stain the same dimer bands in pool II, suggesting heterodimers composed of α2(IV)NC1 and α4(IV)NC1. Dimers (D) and monomers (M) are indicated in panel b, and the four different α(IV)NC1 monomers are also shown.

**FIG. 5.** Schematic illustration of α(IV) chain composition of the three NC1 hexamer subclasses that were recovered by affinity chromatography.

The NC1 hexamers were first separated on the anti-α1(IV)NC1 column, and unbound and bound fractions were then separately applied to the anti-α3(IV)NC1 column (not shown). An attempt to separate the populations further by reversed phase HPLC failed, but the three populations had different retention times, demonstrating differences in their chemical composition (not shown).

**DISCUSSION**

The NC1 hexamers, derived from the C-terminal domains of two cross-linked type IV collagen molecules, were separated
into three distinct populations using two affinity chromatography columns. The first population, a hexamer composed of α1(IV)NC1 and α2(IV)NC1, passed through the anti-α3(IV)NC1 affinity column, but bound to the anti-α1(IV)NC1 affinity column. No α3(IV)NC1 or α4(IV)NC1 were detected in this population. Immunoblot analysis showed that the α1(IV)NC1 subunits were bound in homodimers, (α1(IV)NC1)2, and (α2(IV)NC1)2, indicating that this hexamer population originated from two classical type IV collagen molecules. This population represented about 69% of the NC1 hexamers in GBM.

The second population was found in the material that bound to the anti-α3(IV)NC1 affinity column but not to the anti-α1(IV)NC1 affinity column. It was composed of α3(IV)NC1 and α4(IV)NC1; no α1(IV)NC1 or α2(IV)NC1 were detected. The subunits in this hexamer population were also connected in homodimers, (α3(IV)NC1)2 and (α4(IV)NC1)2, shown by immunoblot analysis. This shows that there is a subclass of the type IV collagen molecule composed of α3(IV) and α4(IV) chains. The population represented about 12% of the NC1 hexamers in GBM.

The third hexamer population, which bound to both affinity columns, was composed of all four α(IV)NC1 subunits and represented about 19% of the NC1 hexamers in GBM. Immunoblot analysis showed they were heterodimers, α1(IV)NC1 connected to α3(IV)NC1 and α2(IV)NC1 connected to α4(IV)NC1.

Immunofluorescence studies have shown that GBM is composed mainly of α3(IV) and α4(IV) chains, while mesangium, Bowman's capsule, and tubular basement membrane contain α1(IV) and α2(IV) chains (7). Our preparation contained smaller amounts of the α3(IV) and α4(IV) containing subclasses than expected from immunofluorescence. This could be explained by the presence of mesangium, Bowman's capsule, and tubular basement membrane in the GBM preparation or that immunofluorescence overestimates the content of α3(IV) and α4(IV) in the GBM.

By analogy to the classical type IV collagen molecule, composed of two α1(IV) chains and one α2(IV) chain, and with the knowledge of the heterodimers α1(IV)NC1-α3(IV)NC1 and α2(IV)NC1-α4(IV)NC1, it is likely that the new type IV collagen molecule is composed of two α3 and one α4 chain.

The existence of homohexamers of α1(IV)NC1 or α3(IV)NC1 or of a heterohexamer composed of only α1(IV)NC1 and α3(IV)NC1, cannot be dismissed because they also bind to the columns. However, homohexamers of α2(IV)NC1 or α4(IV)NC1 or heterohexamers of α2(IV)NC1 and α4(IV)NC1 are less likely to exist since none were found in the material that passed through both affinity columns.

Saus et al. (15) have with immunoprecipitation experiments separated one population of NC1 hexamers enriched in α1(IV)NC1 and a second enriched in α3(IV)NC1, but in both types α1(IV)NC1 and α3(IV)NC1 coexisted. They also found indications for the existence of heterodimers of α1(IV)NC1 and α3(IV)NC1. By N-terminal sequence analysis Gunwar et al. (16) determined the chain origins of dimeric subunits from GBM NC1 hexamers. Their results showed that the α1(IV)NC1 to α4(IV)NC1 chains all were connected in homodimers. They also found a small amount of heterodimers of α1(IV)NC1-α3(IV)NC1. These results support our conclusion that the majority of NC1 hexamers are composed of homodimers. In addition, we can show the existence of α1(IV)NC1-α3(IV)NC1 heterodimers, and of a heterodimer of α2(IV)NC1-α4(IV)NC1.

The distribution of the α5(IV) chain has not yet been shown. It might colocalize with the other chains, show a new distribution, or cross-link to another unidentified α1(IV) chain. The α3(IV) and α4(IV) chains are present only in specialized basement membranes, and their distributions are altered in pathological conditions, suggesting that they play important functional roles. GBM of patients with Alport's syndrome lack α5(IV) and α4(IV) chains (17) and these patients have mutations in the α5 gene. Their GBM is not cross-linked normally. Patients with the autoimmune disorder Goodpasture syndrome have auto-antibodies to the α3 chain that damage basement membranes of their kidneys and lungs (16). These findings underline the need for elucidation of the molecular organization of basement membranes in order to understand the mechanisms by which diseases affect various tissues.

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