Evidence for Separate Networks of Classical and Novel Basement Membrane Collagen

CHARACTERIZATION OF \( \alpha_3(IV) \)-ALPORT ANTIGEN HETERODIMER*

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The COOH-terminal non-collagenous domains (NC1) of type IV collagen from glomerular basement membranes (GBM), lens capsule basement membranes, and Descemet's membrane varied in the distribution of their NC1 subunits. All of these basement membranes (BMs) contained both classical (\( \alpha_1(IV) \) and \( \alpha_2(IV) \)) and novel collagen chains (\( \alpha_3(IV) \), \( \alpha_4(IV) \) and the Alport antigen). Whereas GBM had a predominance of disulfide-bonded subunits, the lens capsule and Descemet's membrane were primarily monomeric, differences that are likely related to the functional and structural diversity of collagens in various tissues. A heterodimer formed from monomeric subunits of \( \alpha_3(IV) \) and the Alport antigen exists in human and bovine GBM. This dimer represents an important cross-link of the NC1 domain of novel collagen. Additionally, immunoaffinity methodology showed that the novel BM collagen hexamers segregate into populations containing only novel BM subunits without the participation of the classical subunits (\( \alpha_1(IV) \) and \( \alpha_2(IV) \)). These data provided evidence for the presence of two separate networks of BM collagen: one containing \( \alpha_1(IV) \) and \( \alpha_2(IV) \), and the other consisting of the novel collagen chains.

Type IV collagen provides the structural framework for specialized sheets of extracellular matrix known as basement membranes (BM).

1. Intact \( \alpha_1(IV) \) and \( \alpha_2(IV) \) molecules of 185 and 170 kDa, respectively, form heterotrimers of helical collagen in a ratio of 2:1 (1). The collagen IV triple helix is joined to three additional helices at their NH\(_2\) termi (2), and the COOH terminus interacts with a second collagen helix at its COOH terminus (3), thus forming a network of type IV collagen similar to chicken wire. At the COOH terminus of each \( \alpha_1(IV) \) and \( \alpha_2(IV) \) chain are non-collagenous domains (NC1) of 26 and 24 kDa, respectively. Homodimerization of these NC1 domains occurs via intermolecular disulfide cross-linking, integrating the COOH termini into the collagen network. When type IV collagen NC1 is isolated from collagenase digests of Englebreth-Holm-Swarm mouse tumor matrix, (4) dimeric and monomeric NC1 subunits are observed on SDS-PAGE gels (5).

In a number of specialized BMs, including the glomerular basement membrane (GBM), this simplistic model of type IV collagen network is complicated by the recognition in collagenase digests of two 28-kDa NC1 monomers derived from additional collagen chains (6, 7). Two-dimensional gel analysis of human GBM reveals a charge distinction between the two peptides (M26\(^+\), pH > 9.0, M28\(^+\), pH 7.0) whereas the two bovine GBM peptides are closer in charge (pH 7.5 and 7.0, respectively) (6). Amino-terminal sequences of purified bovine monomers established the identity of all four monomers and revealed certain amino acid similarities between \( \alpha_1(IV) \), \( \alpha_2(IV) \), and the two 28-kDa peptides (8). This has led to the designation of the peptides M28\(^+\) and M28* as derived from \( \alpha_3(IV) \) and \( \alpha_4(IV) \), respectively (9, 10). The \( \alpha_3(IV) \) NC1 is the primary target of Goodpasture autoantibodies (11) although the sera from patients with this disease react with all NC1 peptides on Western blots (6). Monoclonal and polyclonal antibodies with specificity for NC1 of \( \alpha_1(IV) \), \( \alpha_2(IV) \), \( \alpha_3(IV) \), and \( \alpha_4(IV) \) identify homodimers of \( \alpha_1(IV) \), \( \alpha_2(IV) \), and \( \alpha_4(IV) \) (12). Dimers which react with \( \alpha_3(IV) \) antibodies are not as cationic as the reactive monomer. Recently, the cDNA sequence of the \( \alpha_3(IV) \) NC1 has been determined and compared with that of \( \alpha_1(IV) \) and \( \alpha_2(IV) \) shows a 71 and 61% amino acid homology, respectively, with perfect alignment of cysteine residues characteristic of type IV collagen NC1 (13).

An additional NC1 peptide is detected using an alloantibody obtained from an Alport patient who developed anti-GBM nephritis post-transplantation (14). The reactive 28-kDa NC1 peptide (Alport antigen), more recently characterized using a monoclonal antibody (mAb A7) with identical specificity, is similar in size and charge to \( \alpha_1(IV) \), but can be separated from \( \alpha_1(IV) \) using immunoaffinity chromatography. The Alport antigen, \( \alpha_3(IV) \) NC1, and \( \alpha_4(IV) \) NC1 colocalize in the lamina densa of normal GBM using immunofluorescence microscopy and are absent from the GBM of male Alport patients (16, 17) whose GBM is laminated and split when analyzed by electron microscopy (18–21). Like the Alport alloantibody, mAb A7 discriminates the X-linked mode of Alport antigen inheritance using immunofluorescence analysis of normal, homozygous, and heterozygous Alport GBM and epidermal basement membrane (16).

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‡ The abbreviations used are: BM, basement membranes; LC, lens capsule; GBM, glomerular basement membranes; DM, Descemet's membranes; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; mAb, monoclonal antibodies; PBS, phosphate-buffered saline; NEPHGE, non-equilibrium pH gradient electrophoresis.

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A cDNA sequence with a 83% amino acid homology to α1(IV) NC1, but with gene localization to the Alport locus on the X chromosome, has been designated as α5(IV) NC1 (22). Mutations have been described in 3 out of 18 families tested by restriction fragment length polymorphism (23, 24). A monoclonal antibody to a nonconsensus peptide of α5(IV) stains normal kidney in a distribution similar to that of α3(IV), α4(IV), and the Alport antigen, but, in common with the latter three chains, is not detected in Alport kidney (22). Because the α5(IV) NC1 and Alport antigen NC1 peptide identified by mAb A7 are similar in size and charge, we have made considerable efforts to establish the identity of the Alport antigen and its relationship to α5(IV). Although the tissue distributions, absence from Alport GBM, and two-dimensional immunoblots of α5(IV) and the Alport antigen show identity, the necessary amino acid and nucleotide sequence data for each is unavailable at this time. At this time, we feel it is necessary to distinguish between α5(IV) and the Alport antigen identified by mAb A7 and the Alport alloantibody since the existence of two very homologous proteins has not been ruled out.

In this report, we have analyzed the subunit composition of three basement membranes: human and bovine GBM, bovine lens capsule (LC), and bovine Descemet's membrane (DM), all of which contain α3(IV), α4(IV), and the Alport antigen, as well as α1(IV) and α2(IV) (12). A heterodimer common to these EMs was formed by monomers of α3(IV) and the Alport antigen and did not contain α1(IV) or α2(IV). Further, immunopurified hexamers segregated into disparate populations supporting the presence of two distinct networks of type IV collagen.

MATERIALS AND METHODS

Isolation of Basement Membranes—Fresh bovine renal cortices were diced and homogenized in 0.15 M NaCl using a Brinkmann Polytron (Brinkmann Instruments Co, Westbury, NY) at setting 4.5. Glomeruli were retrieved by size-selective sieving (25). Anterior and posterior capsules were removed from lens of bovine eyes obtained from Pel-Freeze Biologicals (Rogers, AR). Descemet's membrane was peeled from the posterior corneal surface using blunt forceps after freeze/thawing of dissected bovine corneas. Human GBM was obtained as previously reported (6). The collagenase solution used in the isolation and extraction of the GBM, LC, and DM contained the protease inhibitors 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM N-ethylmaleimide, and 1 mM ε-aminocaproic acid (inhibitor buffer). All stages of the preparation were kept on ice. Glomeruli were washed in PBS and disrupted by sonication in 1 M NaCl using 20-s bursts until whole glomeruli could not be found in the solution. GBM was retrieved by centrifugation, and washed extensively with PBS. LC and DM were also sonicated in 1 M NaCl to fragment the BM.

Preparation of Intact NC1 Hexamers—Washed BMs from GBM, LC, and DM were extracted for 24 h at 4 °C using 0.5 M NaCl in PBS (0.15 M NaCl, 0.01 M phosphate, pH 7.4), with protease inhibitors. Membranes were retrieved by centrifugation and a second 24-h extraction in 0.5 M NaCl, PBS was performed. Remaining membranes were then extracted for 72 h at 4 °C using 3% acetic acid containing 10 μg/ml pepstatin A, retrieved by centrifugation, dialyzed, and lyophilized (6). Extracted membranes were digested with type VII bacterial collagenase (Sigma) in 0.05 M Tris, 0.2 M NaCl, 0.002 M CaCl2, pH 7.4, using a Bio-Rad vertical blotting apparatus, and dialyzed against distilled water. Membranes were dialyzed against distilled water and lyophilized.

Preparation of Bovine NC1 Dimers—Bovine GBM was extracted for 48 h in 6 M guanidine HCl, 0.05 M Tris, pH 7.4, retrieved by centrifugation, and washed extensively with 0.05 M Tris, 0.15 M NaCl, pH 7.4. The BMs were then dialyzed against distilled water and lyophilized. Membranes prepared in this way were then suspended in collagenase buffer and digested as described above.

Collagenase-digested bovine GBM was applied to a Sepharose-Q fast flow column (Pharmacia LKB Biotechnology Inc.) run in 0.5 M Tris, 4 M urea, pH 7.4. The unbound fraction containing the NC1 was pooled, dialyzed against distilled water, and lyophilized.

Unbound fractions from Sepharose-Q columns were separated into monomer and dimer pools by gel filtration using a S900 column (2.5 × 100 cm) (Pharmacia Biotech) in 0.05 M guanidine HCl, 0.05 M Tris, pH 7.4. Pools of monomers and dimers were concentrated by evaporation and dialyzed into TBS for immunoprecipitation experiments. Dimer preparations were described in detail elsewhere (26).

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Gel Electrophoresis—To analyze NC1 subunit composition and immunoprecipitated proteins, 40 μg of bovine GBM, LC, and DM or the precipitation extract was applied to 8–18% SDS-polyacrylamide gel (PAGE) and electrophoresed using a discontinuous buffer system (27). 100–400 μg of human and bovine GBM, and bovine LC and DM NC1 were applied to each nonequilibrium pH gradient electrophoresis (NEPHGE) tube gel and electrophoresed for 2750 V h as described elsewhere (6, 28). NEPHGE gels were separated in the second dimension by electrophoresis in 8–18% SDS-PAGE gels. Gels were either stained with Coomassie Blue dye or used for immunoblotting.

Immunoblotting—NC1 proteins were electrophoretically transferred from one or two-dimensional gels to 0.45-μm Immobilon sheets (Millipore Inc., Bedford, MA) in a Bio-Rad vertical blotting apparatus using 100 mA overnight as previously described (29). The transfers were blocked with 1% bovine serum albumin in TBS for 1 h at 37 °C, then reacted with mouse monoclonal antibodies for 1 h at room temperature. Antibodies used, with their specificities, included mAb 10A5, αNC1 (Biocarriecs, Paris, France), mAb A2 (α4(IV)), mAb A10 (α5(IV)), and mAb A7 (the Alport antigen). All antibodies specific to these antibodies have been established in previous studies (12, 14, 17, 30–32). Bound primary antibodies were detected by incubation with alkaline phosphatase-conjugated affinity purified goat F(ab')2 anti-mouse IgG (Caltag, South San Francisco, CA) followed by application of the alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphosphate/nitro blue tetrazolium (Promega Biotech, Madison, WI) in 0.1 M sodium carbonate, 0.001 M MgCl2, pH 9.8. Alternatively, immunoblots were placed immediately in coldfold gold total protein stain (Bio-Rad) and reacted for up to 1 h at 23 °C.

RESULTS

Subunit Composition of Bovine NC1 from GBM, LC, and DM Purification and Analysis of NC1 Hexamers—Collagenase digests of bovine GBM, LC, and DM, and human GBM were separated by nondenaturing gel filtration chromatography. In all preparations, NC1 hexamers (noncovalently associated NC1 dimers and monomers) eluted in a position typical for the 160-kDa complex. Portions of these bovine NC1 preparations were lyophilized and applied to a SDS-PAGE gel.
which was subsequently stained with Coomassie Blue dye (Fig. 1). The GBM had the highest proportion of NC1 disulfide-bonded dimeric subunits while the LC and DM contained primarily NC1 monomers.

**Two-dimensional Gel Analysis of NC1 from Human and Bovine GBM and Bovine LC and DM**—The lyophilized NC1 was further analyzed using two-dimensional NEPHGE gels and immunoblotting with mAbs for subunit identification. In the first experiment, human GBM was compared with bovine GBM (Fig. 2). Anti-α(IV) identified similar monomeric and dimeric subunits of pH 8.0 in both human and bovine NC1. These subunits, and those of α2(IV), pH 6.0, are quite prominently stained by Coomassie Blue dye, whereas staining of the novel NC1 subunits was quite weak. As previously observed, the α3(IV) subunits had a cationic migration in human NC1 (pH > 9.0) while those of bovine NC1 were neutral (pH 7.0). The bovine α3(IV) NC1 dimers appeared to consist of six heterogeneous subunits while those of human NC1 consisted of three subunits. The subunits of the Alport antigen were more complicated than those of α3(IV). In human GBM, mAb A7 recognized the Alport antigen, a 26-kDa monomer of pH 8.0 and homodimers of this monomer, as well as cationic dimers which were also identified with antibody to α3(IV). A similar picture resulted from the staining of bovine GBM. However, the dimers which also react with anti-α3(IV) antibodies were more difficult to distinguish because of overlapping Alport antigen homodimers in this pH range.

In two-dimensional blots of bovine LC and DM (Fig. 3), migration was similar to the NC1 components of GBM. Coomassie Blue staining of gels confirmed the presence of α2(IV) NC1 monomers and dimers. We observed rather weak reactivity of mAb 10 (anti-α1(IV) NC1) with the monomer and homodimeric subunits of α1(IV) NC1. The α4(IV) NC1 which was detected with mAb 85 was overwhelmingly monomeric in subunit composition. LC and DM both contained monomeric subunits of α3(IV) and a heterogeneous group of dimers similar to the bovine GBM, with three migrating beyond the monomers in the pH gradient. mAb A7 (the Alport antigen) was extremely reactive with the LC NC1, even at 50 μg of applied antigen and the excessive proportion of monomers made analysis of that region difficult. However, the dimers appeared identical to those in the bovine GBM with recognition of the α3(IV) containing dimers. Reactivities of mAbs with DM were identical to those with LC. More discreet banding facilitated identification of the dimers reactive with anti-α3(IV) (mAb A2) and anti-Alport antigen (mAb A7).

In summary, the two-dimensional gel analysis revealed monomers and homodimers of α1(IV), α2(IV), α3(IV), and the Alport antigen. The bovine NC1 dimers containing α3(IV) were far more complex than their human counterparts and may contain α3(IV) homodimers in addition to the α3(IV)-Alport antigen heterodimers. Most importantly, this heterodimer of α3(IV) and the Alport antigen was present in all three bovine BMs and in the human GBM.

**Heterodimer Analysis Using Immunoprecipitation**—Bovine GBM NC1 dimers purified by denaturing chromatography, and concentrated by evaporation, were used in SAC immuno-

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**Fig. 1.** SDS-PAGE gel of NC1 fractions from Bio-Gel A 0.5-m column separations of bovine GBM (1), LC (2), and DM (3), stained with Coomassie Blue dye. Whereas the NC1 of GBM is primarily dimeric in composition, that of LC and DM is composed principally of monomeric NC1 subunits.

**Fig. 2.** Two-dimensional gel analysis of NC1 from human (h) (left panel) and bovine (b) (right panel) GBM. Western blots were reacted with mAbs as indicated. A and E, anti-α3(IV) NC1 (mAb A2). B and F, anti-Alport antigen (mAb A7). C and G, anti-α1(IV) NC1 (mAb 10). D and H, Coomassie Blue dye. Note that α3(IV) dimers have a cationic (pH > 9.0) migration and consist of three subunits (A, arrows) in human NC1, whereas bovine dimers were neutral and appeared to contain five to six subunits (E). The human cationic monomer reacts weakly. The anti-Alport antigen (B) reacts in the human system with 26-kDa monomeric subunits and associated homodimers as well as cationic dimers (arrow) similar in size, charge, and location to the dimers of α3(IV) NC1 in A. The more neutral bovine α3(IV) NC1 dimers overlap in reactivity with the Alport antigen (F) and are not as clearly analyzed as human dimers. The monomers and dimers of α1(IV) NC1 in human (C) and bovine (G) collagen are similar except for the greater reactivity of the human 26-kDa monomeric subunits. Gels stained with Coomassie Blue dye (D and E) are shown for comparison.

**Fig. 3.** Two-dimensional gel analysis of NC1 from bovine (b) lens capsule (LC) and Descemet’s membrane (DM). Blots were reacted with mAbs as indicated. A and E, anti-α1(IV) NC1 (mAb 10). B and F, anti-α4(IV) NC1 (mAb 85). C and G, anti-α3(IV) NC1 (mAb A2). D and H, anti-Alport antigen (mAb A7). The monomers and dimers react weakly with anti-α1(IV) NC1 (A and E). Note the predominance of monomeric components of α4(IV) NC1 (B and F) and Alport antigen (D and H) in contrast to α3(IV) (C and G). Homodimers of α3(IV) NC1 and heterodimers of α3(IV) NC1-Alport antigen (arrows) are apparent.
n proud precipitations. 200 μl of antigen were incubated with either anti-α3(IV) NC1, anti-Alport antigen, or anti-α1(IV) NC1. Following a second incubation with secondary antibodies to facilitate lattice formation, the complexes were allowed to bind to Staphylococcus A cell coat protein A. The complexes were pelleted, washed, extracted with SDS-sample buffer, and separated on a SDS-PAGE gel. Colloidal gold staining was used to stain protein transferred to Immobilon by Western blotting (Fig. 4, left panels) and showed NC1 dimers precipitated by all three antibodies. Immunoblotting of the same precipitates with anti-Alport antigen indicated that dimers precipitated by either anti-Alport antigen or anti-α3(IV) NC1 contained subunits of the Alport antigen while those precipitated by anti-α1(IV) NC1 antibody did not.

To further analyze the heterodimer of α3(IV)-Alport antigen, SAC precipitations using anti-Alport antigen were applied to NEPHGE gels and after electrophoresis in two dimensions were transferred to Immobilon and immunoblotted with anti-α3(IV) and anti-Alport antigen (Fig. 4, right panels). The dimers precipitated with the Alport antibody contained both the α3(IV)-Alport antigen heterodimers of pH 7.0-7.5 and Alport antigen homodimers of pH 7.5-8.0.

**Immunopurification of Intact Bovine NC1 Hexamers**

Affinity purified anti-α3(IV) NC1 (mAb A2) and anti-Alport antigen (mAb A7) were used to construct Affi-Gel HZ hydrazine affinity matrices. Anti-α1(IV) (NC1 (mAb 10) was also used, but this antibody failed to bind antigen when linked to the support system. NC1 from bovine GBM, LC, and DM were subjected to batch purification using these two affinity matrices. It is noteworthy that NC1 isolated for this purpose was not subjected to denaturing conditions during its purification procedure. When experiments were performed with NC1 which had been subjected to 4 M urea, the results indicated that hexamers dissociated in this buffer and reassociated at random. Use of non-denaturing buffers and deletion of the Q-Sepharose step were precautions to keep NC1 hexamers intact until the post-purification extraction step. The presence of Alport antigen monomers in precipitated protein from both matrices confirmed that the noncovalent bond within the hexamers had not been dissociated. The Affi-Gel HZ antibody-antigen complex was thoroughly washed and extracted into SDS sample buffer for analysis by immunoblotting.

When NC1 hexamers of GBM LC and DM were purified using the anti-Alport HZ matrix they contained subunits reactive with both anti-Alport antigen (homodimers and monomers of the Alport antigen and the heterodimer of α3(IV)-Alport antigen) and anti-α3(IV) NC1 monomer and the heterodimer of α3(IV)) (Fig. 5A). The trend in these reactions to favor purification of the Alport antigen subunits was purely stoichiometric, since there are more Alport antigen containing subunits in the NC1. Most importantly, these hexamers did not react with anti-α1(IV) NC1 antibody.

Batch purification of hexamers using the anti-α3(IV) NC1 Affi-Gel HZ hydrazine support (Fig. 5B) gave similar results, identifying NC1 hexamers containing subunits of α3(IV) and Alport antigen, but no subunits of α1(IV). Using the anti-α3(IV) NC1 HZ matrix, α3(IV) subunits were more prominent since the only Alport antigen precipitated with anti-α3(IV) would be in heterodimeric form. NC1 subunits of the α4(IV) chain were found in both experiments but with reactivity too low to be distinguished from the native bovine NC1 hexamers.
Novel BM Collagen Network

The α3(IV)-Alport antigen heterodimer is likely a critical assembly step in the formation of the novel collagen network and is the only heterodimer known to exist in the human NC1. This disulfide bonding may occur between similar helices composed of novel collagen heterotrimers or between helices of novel collagen heterotrimers and Alport antigen homotrimers. Since it will be difficult to prove how many novel networks exist, we will assume that tissue-specific combinations are limited by the cells synthesizing these chains. Nonetheless, formation of this heterodimer has far-reaching implications with respect to Alport familial nephritis. It would appear that most of the α3(IV) NC1 is engaged in a disulfide linkage with the Alport antigen. We have shown that this also occurs in cultured bovine corneal endothelial cells, a reaction that may drive formation of the COOH-terminal cross-link in the network. Absence of the Alport antigen would interfere with formation of hexamers containing the novel heterotrimers, and the related helix as well. Furthermore, in BMs like the GBM, LC, and DM, where α3(IV) is integral to the novel collagen network, absence of α3(IV) itself could result in the Alport phenotype.

What is the role of α5(IV) and how does it relate to the Alport antigen? We have shown that the polyclonal anti-α5(IV) and anti-Alport (mAb A7) antibodies stain the same renal BMs and that neither bind the Alport GBM, indicating complete concordance in tissue reactivity. In addition, Western blotting of NC1 with anti-α5(IV) showed a monomeric and dimeric subunit structure similar to that observed using antibodies to the Alport antigen. However, without biochemical proof that α5(IV) and the Alport antigen are identical, we cannot rule out the presence of a sixth chain of type IV collagen which is very homologous to α5(IV). It is possible that the genes encoding Alport antigen and the α5(IV) are both located at the Alport locus on the X-chromosome, perhaps in a head to head, bi-directional promoter arrangement as was found with the classical collagen chains (34). The α3(IV) and α4(IV) genes may be similarly structured on autosomal chromosome 2 (35).

With respect to BM collagen nomenclature, we wish to note that the designation of the novel collagens as chains of type IV collagen marks the first instance in which collagen molecules which do not form helices or known interactions together have been grouped in the same collagen family, but this information was not available when the chain nomenclature was designated. Homology of the known NC1 amino acid sequences range from 60 to 83% and cysteine residues are in perfect alignment, which is compelling evidence for their relatedness (22, 36, 37). However, the demonstrated difference between the classical and novel collagen IV molecules with respect to ontology, tissue localization, alterations in disease, and chain interactions, all provide strong support for two distinct BM collagen networks. Previous studies of the distribution of these components in the glomerulus suggest that each network is assembled independently by distinct and separate populations of cells (12, 14, 16, 17, 26, 30-32). In this regard, the classical collagen chains are formed by mesangial/subendothelial cells whereas the novel network is derived from the visceral epithelium. These differences are amplified in both hereditary and acquired disease.

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