Assembly of Type IV Collagen

INSIGHTS FROM α3(IV) COLLAGEN-DEFICIENT MICE*

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Type IV collagen includes six genetically distinct polypeptides named α1(IV) through α6(IV). These isoforms are speculated to organize themselves into unique networks providing mammalian basement membranes specificity and inequality. Recent studies using bovine and human glomerular and testis basement membranes have shown that unique networks of collagen comprising either α1 and α2 chains or α3, α4, and α5 chains can be identified. These studies have suggested that assembly of α5 chain into type IV collagen network is dependent on α3 expression where both chains are normally present in the tissue. In the present study, we show that in the lens and inner ear of normal mice, expression of α1, α2, α3, α4, and α5 chains of type IV collagen can be detected using chain-specific antibodies. In the α3(IV) collagen-deficient mice, only the expression of α1, α2, and α5 chains of type IV collagen was detectable. The non-collagenous 1 domain of α5 chain was associated with α1 in the non-collagenous 1 domain hexamer structure, suggesting that network incorporation of α5 is possible in the absence of the α3 chain in these tissues. The present study proves that expression of α5 is not dependent on the expression of α3 chain in these tissues and that α5 chain can assemble into basement membranes in the absence of α3 chain. These findings support the notion that type IV collagen assembly may be regulated by tissue-specific factors.

Type IV collagen is a family of complex polypeptides and a major constituent of mammalian basement membranes (1, 2). The α1(IV) and α2(IV) chains are products of distinct genes located pairwise in a head-to-head fashion on chromosome 13 in humans (3). The α3(IV) and α4(IV) chains are present in the same orientation on chromosome 2, and the α5(IV) and α6(IV) chains are located on the X chromosome in humans (4). The type IV collagen protomer (trimmer) consists of three α chains that come together through associations among their NC1 globular domains followed by folding of the collag enous domains into triple helices through covalent and non-covalent interactions (1, 2, 4, 5). The protomer is divided into three different domains: an NH2-terminal 7 S domain, a middle triple-helical domain, and a COOH-terminal NC1 globular domain (1, 4, 5). With six different α chains known at present, 56 different combinations of triple-helical protomers are possible (1, 2, 4–6). Transplantable, basement membrane-producing mouse Engelbreth-Holm-Swarm sarcoma (EHS) tumor is a plentiful source of type IV collagen (2). Type IV collagen isolated from EHS tumor contains only α1 and α2 chains (7). These chains were identified to exist as an α1α2 type IV collagen protomer in a 2:1 ratio (1, 2, 4–6). By use of rotary shadow electron microscopy it could be shown that four protomers of type IV collagen are connected by association of the 7 S domain forming a spider-shaped structure (1, 2, 4–6). Each protomer is bonded with another protomer by its NC1 domain to form interlocking hexamers (6). The combination of these two types of interaction along with extensive side-by-side (lateral) associations within the collagenous triple helix allows the formation of a network that serves as a scaffold for the basement membrane (6). Type IV collagen protomer compositions in the human basement membranes are still largely unknown.

Most of our current understanding of the structure and self-aggregating properties of type IV collagen is derived from studies using mouse EHS tumor, which contains only α1 and α2 chains (1, 2, 4–6). Recently, all six chains of type IV collagen were identified by protein biochemistry techniques using bovine testis and kidney GBM (8, 9). In these studies, pseudolysin (Pseudomonas elastase) at two different temperatures was shown to extract two different populations of type IV collagen selectively (8, 9): one population that just contains α1 and α2 chains, like the EHS tumor, and another that contains α1, α3, α4, α5, and α6 chains (8, 9). These reports speculate that type IV collagen networks involving the α3, α4, and α5 chains may play a unique role in the specialized basement membranes such as seminiferous tubule basement membrane (STBM) and GBM (8, 9). Additionally, recent experiments have shown that in most cases, α5 chain mutations in Alport syndrome (an X-linked hereditary kidney disease) lead to an absence of α3 chain of type IV collagen in the GBM of these patients (10–17). These studies, along with immunoprecipitation experiments using human GBM hexamers, have led to the suggestion that α5 and α3 are regulated tightly in their expression and are dependent on each other to form protomeric network of type IV collagen (9, 17, 18).

Identification of new chains of type IV collagen with their restricted tissue distribution and the presence of separate net-brane; STBM, seminiferous tubule basement membrane; WT, wild type; α3KO, α3 type IV collagen-deficient mice; ELISA, enzyme-linked immunosorbent assay; LBM, anterior lens capsular basement membrane.

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works raises a number of unanswered biological questions pertaining to the assembly of these polypeptides (3). It is important to determine the organization of newly discovered human type IV collagen α chains in forming protomers (trimers) and to determine how these new molecules might contribute to the structural and functional diversity of basement membranes. In the present study, we isolated type IV collagen from the α3 collagen-deficient mice and their wild type (WT) littermates and evaluated their type IV collagen α chain composition and NC1 hexamer organization.

MATERIALS AND METHODS

All chemical reagents, horseradish peroxidase, and alkaline phosphatase-conjugated antibodies toward mouse, rabbit, and human IgG were purchased from Sigma and Fisher Scientific. Type IV collagen-specific antibodies were generated and specificity determined as described previously in other publications (9, 19–23).

The α3 collagen-deficient mice were generated as previously (24, 25). Kidneys, lungs, and lens, and inner ears were isolated from 3KO mice and heterozygote mice immediately after the sacrifice of the mice, and the tissue was snap frozen immediately in liquid nitrogen for type IV collagen extraction. For studies involving immunofluorescence, the tissue was processed as described previously (24). SDS-PAGE, Western blotting, and direct ELISA were performed as described previously (19–24, 26–30). For Western blotting, biotin-labeled anti-rabbit IgG was used as the second (detection) antibody.

Immunohistochemical Staining—Immunohistochemical detection of type IV collagen chains was performed as described previously (24). These antisera were produced using synthetic peptides corresponding to regions of the mouse NC1 domains homologous to the sequence used (31) to produce type IV collagen α chain-specific antibodies for the human chains. Antisera were tested by ELISA using plates precoated with either the synthetic peptide or recombinant NC1 domains. All antisera were specific for the type IV collagen α chains to which they were raised (24).

Fresh frozen tissues (with exception of the cochlea) were sectioned at 5 μm and collected onto poly-L-lysine-coated slides. Cochleas were perfused with Carnoy’s fixative, decalcified, embedded, and cut at 8 μm as described previously (24). Slides were postfixed by immersion in cold 95% ethanol for 15 min and allowed to air dry. Prior to staining, sections were first immersed by immersion in 6.0 M urea and 0.1 M glycine (pH 2.5) for 1 h at 50 °C. Following three 10-min washes in phosphate-buffered saline, primary antibodies were applied in 7% w/v non-fat dry milk (Bio-Rad) and allowed to react for 2 h at room temperature in a humidified chamber. After four 10-min washes with phosphate-buffered saline, fluorescein isothiocyanate-conjugated secondary antibodies were applied (Vector Laboratories) and allowed to react for 1 h at room temperature. Slides were then washed in phosphate-buffered saline four times at room temperature, and a small amount of Vectashield mounting medium was applied (Vector Laboratories) before sealing the specimen under glass coverslips using clear nail polish. Immunofluorescence was visualized using an Olympus BH-2 fluorescence microscope configured with Cytovision Ultra (Applied Imaging, Inc.) image analysis software.

Immunoprecipitation Experiments—The NC1 hexamer was prepared by bacterial collagenase and analyzed by SDS-PAGE and immunoblotting as described previously (19–23, 26–30, 32). Immunoprecipitation was performed using the polyclonal α1, α3, and α5 antibodies and the methods described by Klepeel et al. (18) and Johansson et al. (33), with some modifications. Briefly, 10 mg of collagenase digest of detergent-extracted inner ear, lens, and kidney GBM from WT and knockout mice were incubated with antibodies to the α chains, and the resultant mixture was precipitated with antibodies to rabbit IgG conjugated to protein A and analyzed by SDS-PAGE, immunoblotting, and ELISA.

Statistical Analysis—All values are expressed as mean ± S.E. Analysis of variance with a one-tailed Student’s t test was used to identify significant differences in multiple comparisons. A level of p < 0.05 was considered statistically significant.

RESULTS

Immunofluorescence analysis was used to examine α chain composition, in control versus 3KO mice, of the STBM; the GBM; the basement membranes of the inner ear, including the strial capillary basement membrane; and the lens capsule. As illustrated in Fig. 1 and reported previously for STBM (9) and bovine kidney (8), normal mice contain all five chains (Fig. 1, WT, Kidney, and Testis), whereas in mutant mice, collagen α1(IV) and α2(IV) chains persist in STBM, but collagen α3(IV), α4(IV), and α5(IV) chains are absent (Fig. 1, α3KO, Testis). This same pattern of expression is observed in the GBM and the basement membranes of the renal tubules of normal versus mutant mice (Fig. 1, α3KO, Kidney), consistent with earlier reports (12–14).

However, in the anterior capsular material of the lens and the strial capillary basement membranes, the scenario is different. Here, in the α3KO mice, the α5(IV) chain continues to be assembled in the absence of α3(IV) and α4(IV) chains (Fig. 2). In the inner ear, mid-modular cross-sections reveal that the track of basement membrane running from the limbus, down the inner sulcus, across the basilar membrane, up the external sulcus to the spiral prominence, and radiating into the spiral ligament surrounding the root cell processes in tissue from the mutant mouse is devoid of collagen α3(IV), α4(IV), and α5(IV) chains (Fig. 2). In contrast, in the capillaries of the stria
vascularis, the collagen α5(IV) chain is assembled into basement membrane (see structures denoted by arrows in Fig. 2, Inner ear WT and α3KO for α5 antibodies).

We extracted type IV collagen from detergent extracts of anterior lens capsule, cochlea, kidney cortex, and testis by bacterial collagenase from the control and α3KO mice. The collagenase-solubilized material analyzed by SDS-PAGE and Coomassie Blue staining revealed major sets of bands in the region of 46 kDa and 28 kDa (data not shown), consistent with the pattern seen for denatured NC1 hexamer from various tissues (32, 34–37). This material was used (21, 32, 35–37) in direct ELISA experiments using α chain-specific antibodies. The α1, α2, α5, and α6 chains were present in all tissues analyzed (Fig. 3). The α3 and α4 chains were present in the control cochlea, anterior lens capsule, kidney cortex, and the testis, but they were absent in the tissues of the α3KO mice. These results are consistent with the results observed in the immunofluorescence experiments (Figs. 1 and 2). Immunoblotting experiments correlate well with the ELISA experiments. The α1/α2 antibodies show binding to the dimeric (~46 kDa) and monomeric (~28 kDa) forms of the NC1 domain of α1 and α2 chains of type IV collagen in all the tissues analyzed (Fig. 4). The α5 chain was present in all the tissues analyzed except for the α3KO kidney and testis (Fig. 4). The α3 and α4 were detectable only in the WT anterior lens, cochlea, and the kidney and were absent in all tissue analyzed in the α3KO mice (data not shown). These results suggest that α5 expression is present in the inner ear cochlea and anterior lens capsule of the α3KO but is absent in the kidney cortex of the same mice.

To determine the network organization of α5 chain in the α3KO mice, we performed immunoprecipitation experiments utilizing mouse α1 and α5 chain-specific polyclonal antibodies using NC1 hexamer preparations from the anterior lens capsule of the WT and α3KO mice. The specificity of the mouse α1 and α5 chain antibodies was established previously (9, 19–23) and determined again by direct ELISA using recombinant human type IV collagen α1–α6 NC1 domains (data not shown). Using collagenase-solubilized α3KO kidney renal basement membrane, the α1 antibodies immunoprecipitated a 160-kDa band corresponding to the molecular mass of NC1 hexamer (Fig. 5, ND). Under denaturing conditions this band resolved into a set of bands in the range of 46 and 28 kDa (Fig. 5, D). These bands were immunoreactive with α1, α2, and α5 antibodies (Fig. 5). The α3 and α4 antibodies did not reveal any significant binding (Fig. 5). These results suggest that α1 antibodies can precipitate the α2 and α5 NC1 domain in a hexamer form. The α2 antibodies only immunoprecipitated hexamers that contain α1 and α2 NC1 domains (data not shown), suggesting that α5 NC1 does not form hexamers with α2 NC1. The α5 antibodies immunoprecipitate NC1 hexamers that contain only α1 and α5 NC1 domains (Fig. 6). The coprecipitation of α1 and α5 with α5 antibodies suggests that these molecules exist in the same hexamer and hence participate in type IV collagen network formation independently of α2, α3, α4, and α6 chains. When α3 antibodies were used to immunoprecipitate WT lens capsule collagenase-digested material, the immunoprecipitate revealed predominant binding to α3 and α4 chains with minor binding to α1 antibodies (Fig. 7A). These results suggest that, at least in the lens capsule basement membrane,
DISCUSSION

Several reports in the last few years suggest that the expression of α3 chain of type IV collagen is dependent on the expression of α5 chain and vice versa (3, 8, 9, 11–17, 22, 38–43). This suggestion is derived from biochemical and immunological studies using bovine and human kidneys and kidneys from patients with Alport syndrome2 (3, 8, 9, 11–17, 22, 38–46). Immunofluorescence experiments using human kidney sections from normal and Alport patients reveal an absence of α3, α4, and α5 chains of type IV collagen from the GBM of most Alport patients (3, 8, 9, 11–17, 22, 38–45). In some of these X-linked Alport patients, mutations and deletions of α5 chain have been identified as the primary genetic defect (11–16, 45, 47). These studies led to the conclusion that assembly of α3, α4, and α5 chains to form networks of type IV collagen must be regulated tightly, and hence the absence of one of these chains (the α5 in the case of X-linked Alport syndrome) leads to the absence of the other two chains (8, 9, 11–14, 16, 38, 39). Co-regulation of α3 and α5 protomer assembly gained further support when the mRNA levels for these proteins were shown to be unaltered in the human Alport kidneys (48).

Biochemical studies involving immunoprecipitation with α chain-specific antibodies using the NC1 hexamer from bovine and human GBM type IV collagen reveal an association of α3 and α5 NC1 domains in the same hexamer. These results validate further the notion that the expression of α3 and α5 is regulated tightly in basement membranes, especially the GBM (18). In a recent study, pseudolysin was used to extract selectively different networks of type IV collagen at different temperatures. At 4 °C, the enzyme selectively extracted a complex of α1- and α2-containing type IV collagen proteins. Interestingly, when the resultant pellet from the earlier digestion was re-extracted with pseudolysin at 25 °C, a complex of proteins enriched in the α3, α4, and α5 was extracted. These results led the authors to propose the existence of two networks of type IV collagen: one that is composed of α1- and α2-containing type IV collagen proteins and another that contains predomi-

2 Alport syndrome is predominantly an X-linked hereditary kidney disease associated with progressive renal failure leading to end stage renal disease, sensorineural deafness, and ocular lesions. The genetic basis of the disease has been identified as mutations and deletion in the GBM-specific collagen isoform, the α5 chain.
Fig. 7. Immunoprecipitation of collagenase-solubilized WT LBM by α3 and α5 antibodies. Panel A, anti-α3 antibodies were used to immunoprecipitate WT collagenase-solubilized LBM (10 mg), and the immunoprecipitate was analyzed by direct ELISA. The α3 antibodies were used at a dilution of 1:200 for immunoprecipitation analysis. Panel B, anti-α5 antibodies were used to immunoprecipitate WT collagenase-solubilized LBM (2 mg), and the immunoprecipitate was analyzed by direct ELISA. The α5 antibodies were used at a dilution of 1:200 for immunoprecipitation analysis. Immunoprecipitation was performed as described under “Materials and Methods.” Direct ELISA is shown as a bar graph. α1, α2, α3, α4, and α5 denote antibodies against these chains.

Fig. 8. Schematic illustration of NC1 hexamer population in LBM of α3KO mice. Each oval structure represents single α chain NC1 domain. The figure represents the hexamer populations that were identified by immunoprecipitation experiments in the present study. The α1- and α5-containing hexamer, as shown for α3KO LBM, was also identified in the WT mice.

nantly α3, α4, and α5 chains in a highly cross-linked state with interchain associations (8, 9, 48). These results suggest that α3 and α5 are present in the same network of type IV collagen and that their expression and assembly in the kidney are coordinated tightly (8, 38, 39).

Most of the studies relating to α3 and α5 assembly have been performed in the kidney and more recently in the testis (8, 9, 18, 38, 39). In the skin, where α5 is present, α3 is normally absent, suggesting the existence of type IV collagen networks that include the α1 and α2; α1, α2, and α5; or α3 and α6 (8, 9, 11–14, 17, 18, 38, 39, 41). The presence of α3- and α6-containing networks in the kidney GBM is not possible because of the lack of α6 in the GBM, although in the Bowman’s capsule basement membrane α5 and α6 are present in association with α1 and α2 (49).

In this report we present a mouse that lacks the expression of α3 chain at both the mRNA and protein level (24). This mouse provided the opportunity to test the hypothesis of whether assembly of α3 and α5 chains is always regulated tightly in basement membranes in which they are both normally present. In our analysis of various basement membranes in the α3KO mice, we found that in the kidney, a lack of α3 was associated with the absence of α4 and α5, whereas in the anterior lens capsule and the strial capillary basement membrane of the cochlea, the expression of α5 persisted despite the absence of α3 chain in these basement membranes. Our results show that the α5 NC1 colocalized with α1 chain in the same hexamer in the α3KO and WT mice. These results suggest that α1 and α5 can be present in the same network in the absence of α3 chain (Fig. 8). In the kidney, the expression of these two chains seems tightly coordinated and dependent on the expression of each other, but our study presents a possibility of tissue-specific type IV collagen assembly. Such tissue-specific α chain assembly can be achieved presumably via factors such as chaperones. In this regard a heat shock protein (47 kDa) chaperone has been identified for collagen assembly, including the type IV collagen (50–53). We speculate that assembly of type IV collagen α chains in the formation of organ basement membranes is potentially dependent on tissue-specific assembly factors that are yet to be discovered. Such structural differences among organ basement membranes potentially contribute to important functional diversity.

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