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GLOMERULAR BASEMENT MEMBRANE: Evidence for Collagenous Domain of the α3 and α4 Chains of Collagen IV

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SUMMARY: A collagenous component(s) of Mr = 60K was extracted from glomerular basement membrane with urea and was purified. Upon digestion, it yielded a collagenase-resistant fragment(s) of Mr = 23.5K. Both component and fragment showed immunochemical identity with the noncollagenous domains of the new α3 & α4 chains of collagen IV. The component is characterized by a collagenous domain of about 280 residues and a noncollagenous domain of about 250 residues. These findings further establish these new chains as distinct entities of collagen IV.

Collagen IV is the major constituent of renal glomerular basement membrane (GBM). This component exist as a supramolecular structure which binds other macromolecular constituents of GBM, i.e. laminin, heparan sulfate proteoglycan, and entactin (1). The structure of the classical protomer of collagen IV is characterized by three distinct domains (7S, triple-helical, and NC1), and it is composed of two α1- and one α2-chain (1,2). The NC1 (noncollagenous) domain, which connects two adjoining protomers, exists in hexamer form upon excision by collagenase digestion.

Our recent studies of the NC1 hexamer have revealed the existence of two additional chains (α3 & α4) of collagen IV in GBM (3,4). The evidence is based on physicochemical properties of the NC1 hexamer and on immunochemical and chemical properties of hexamer subunits. We postulated that both chains have a collagenous domain on the basis of the extraordinary relatedness of their NC1 domains to those of the classical α1 & α2 chains, including hexamer structure, physicochemical properties, and amino acid sequence of N-terminal region (2). Recently, an α5 chain was discovered using

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molecular cloning technology and was shown to contain both collagenous and noncollagenous domains (5).

In the present study, we report direct chemical evidence for the existence of a collagenous domain for both α3 & α4 chains. These findings further establish these chains as distinct entities of collagen IV.

MATERIALS AND METHODS

Materials: Reagents for electrophoresis were from Bio-Rad and prestained protein markers from BRL. Urea and protease inhibitors were from Sigma Chemical Co. Sepharose was from Pharamacia and DE-52 cellulose from Whatman. The C18 and GF-250 HPLC columns were from Vydac and Dupont, respectively.

Urea Extraction of GBM: Bovine GBM was isolated as previously described (6). GBM (3 g) was suspended at a concentration of 10 mg/ml in 8 M urea, 0.1 M Tris-HCl, 5 mM benzamidine-HCl, 5 mM -aminocaproic acid, 30 mM EDTA, 4 mM N-ethylmaleimide and 0.05 % sodium azide, pH 8.5. Extraction was carried out for 48 h at 37 C. The insoluble portion was removed by centrifugation at 28,000 x g for 30 m at room temperature. The urea-soluble fraction (570 mg) was concentrated by ultrafiltration (Amicon YM-10 filter).

Isolation of 60k Component: Urea-soluble GBM was fractionated on a column of Sepharose Cl-4B (5 X 77 cm) equilibrated in 8 M urea, 0.1 M Tris-HCl, 10 mM EDTA and 1 mg/ml pepstatin A, pH 7.5 at room temperature. Elution proceeded at a flow rate of 50 ml/h and 12 ml fractions were collected. The fractions containing the majority of the GP-reactive material were pooled and dialyzed against 8 M urea, 0.01 M Tris-HCl, pH 8.0, concentrated by ultrafiltration, and then fractionated on a DEAE-cellulose (DE-52) column (2.5 x 12 cm) equilibrated with dialysis buffer. Unbound material was eluted with 860 ml of equilibration buffer. Bound material was eluted in three steps. The first two steps consisted of linear gradients of 0-0.15 M NaCl and 0.15-0.30 M NaCl, each in a total volume of 600 ml equilibration buffer. In the third step, the column was eluted with 0.6 M NaCl in 600 ml of equilibration buffer. The fractions with the highest GP reactivity were pooled, concentrated, and further purified by reversed-phase HPLC on a C18 column using conditions as previously described (7). The 60K component which appeared as a sharp peak was lyophylized, redisolved in 8 M urea and chromatographed on a GF-250 HPLC column, using 0.2 M ammonium acetate, pH 8.5, as the eluent.

Electrophoresis and immunochemical techniques: SDS-PAGE was performed using a linear gradient (6-22%) of acrylamide, and gels were analyzed by silver staining and Western blot analyses, as previously described (6). The antibodies were previously described. Human GP-antibodies and rabbit anti-M2* are directed against the NC1 domain of the α3(IV) chain (3), rabbit anti-M3 against the NC1 domain of the α4 chain (3,4), and anti-M1 against the NC1 domain of the α1 & α2 chains (8).

RESULTS AND DISCUSSION

Western blot analyses of urea-soluble GBM showed the existence of several components of Mr above 50K. The 60K component, was

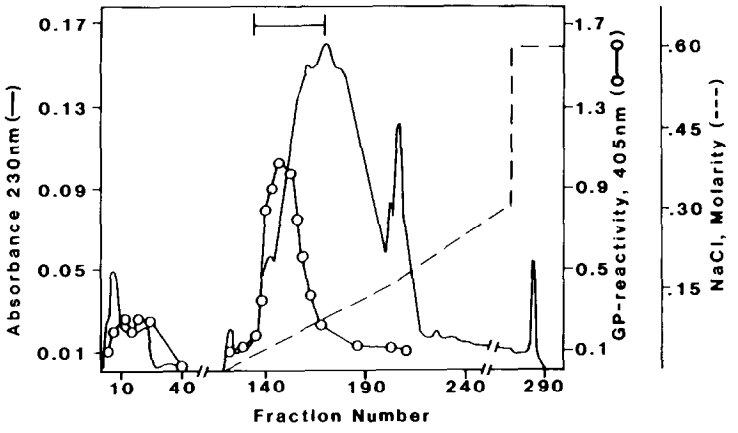

Figure 1. Anion-exchange chromatography of the GP-reactive material. GP-reactive material (80 mg) from the Sepharose CL-4B column were fractionated on the DE-52 column. Elution of protein was monitored by absorbance at 280 nm and elution of GP-reactive material by direct-binding ELISA and expressed as absorbance at 405 nm.

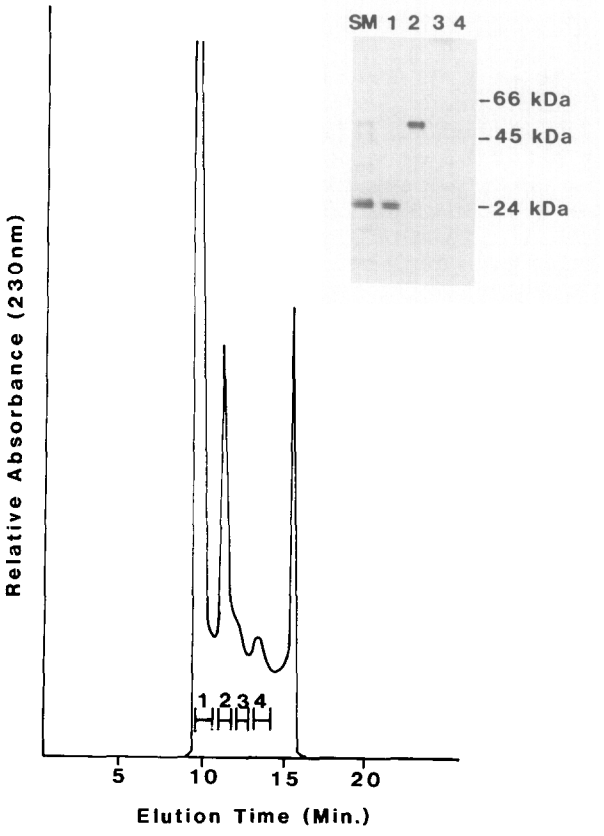

Figure 2. Gel filtration HPLC of the GP reactive material obtained from the reversed-phase HPLC column (Fig. 2). The inset shows the SDS-PAGE patterns (silver stained) of the four fractions. The 60K GP-reactive component eluted in fraction 2.

purified by a combination of chromatographic procedures. The initial extract was fractionated on Sepharose 4B and fraction V which reacted with Goodpasture (GP) sera (data not shown) was pooled and further purified by DEAE-cellulose chromatography (Fig. 1). The GP-reactive fraction was fractionated on an HPLC reversed-phase column (data not shown) and then on an HPLC G-250 column (Fig.2). SDS-PAGE analysis showed a single band with an apparent Mr = 60K (Fig.2, inset), and amino acid analysis revealed that it has a collagen-like characteristic in which it contains substantial amounts of HoPro, HoLys, and Gly (Table I).

Table I

Amino Acid Composition of the 60 kDa Component and Its Noncollagenous and Collagenous Domains

Amino acid	60 kDa Component[a]	α3(IV)NC1 Domain[b]	Collagenous Domain[c]	
	residues/ molecule	residues/ molecule	residues/ molecule	residues/ 1000 res.
HoPro	32.2	1.2	31.0	111
HoLys	4.7	0.4	4.3	15
Gly	111	27.6	83.4	299
Asp	32.2	15.8	16.4	58.8
Thr	26.4	16.2	10.2	36.5
Ser	45.4	23.8	21.6	77.4
Glu	54.4	23.5	30.9	111
Pro	35.9	24.0	11.9	42.6
Ala	29.6	20.5	9.1	33
Val	20.1	10.1	10.0	35.8
Met	9.5	8.1	1.4	5.0
Ile	17.9	11.8	6.1	21.9
Leu	28	19.0	9.0	32.2
Tyr	13.7	7.5	6.2	22.2
Phe	24.8	15.6	9.2	33.0
His	7.4	5.7	1.7	6.1
Lys	10.6	5.2	5.4	19
Arg	25.3	13.4	11.9	42.6

[a]The composition is based on 528 residues/molecule which corresponds to a M_r = 60,000, as determined by SDS/PAGE (9).

[b]The composition is taken from reference 8 and is based on 249 residues/molecule which corresponds to a M_r = 26,100, as determined by SDS/PAGE (9).

[c]The composition of the collagenous domain is the difference between the known composition of the 60 kDa component and that of the α3(IV)NC1 domain, for a total of 279 residues (528-249). The composition of the collagenous domain is essentially the same when calculated on the basis of the composition of either the α3(IV)NC1 or α4(IV)NC1.

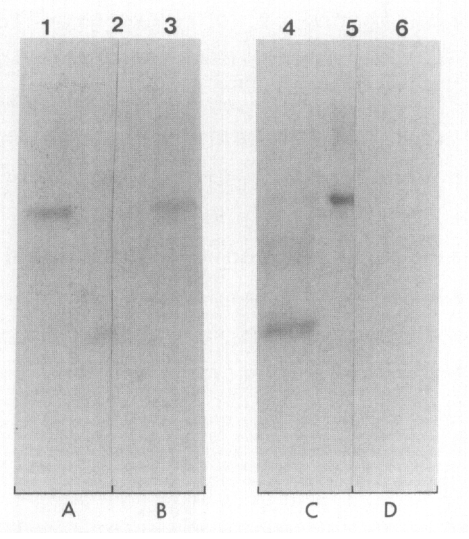

Figure 3. Immunochemical analysis of the 60K component and its collagenase-resistant fragments. Lanes 1,3 & 5 contain purified 60K component, and lanes 2,4, & 6 contain collagenase-digested 60K component. Panel A was stained with GP-antibodies which are directed to the α3(IV)NC1 domain. Panel B was stained with anti-α4(IV)NC1. Panel C was stained with anti-α3(IV)NC1. Panel D was stained with anti-α1&α2(IV)NC1. Lane 2 was divided and one-half stained with GP-antibodies and the other with anti-α4(IV)NC1. Likewise, lane 5 was divided and one-half stained with anti-α3(IV)NC1 and the other with anti-(α1- + α2(IV)NC1).

Upon collagenase digestion, this component yielded a lower molecular weight fragment of apparent Mr = 23.5K (Fig.3). Both the component and its collagenase-resistant fragment reacted with Goodpasture antibodies and polyclonal antibodies (lanes 2 & 3), which are directed against the NC1 domain of the α3 chains, and with polyclonal antibodies against the α4 chain (lanes 4 & 5), but not with polyclonal antibodies directed against the α1 chain. The size of the collagenase-resistant fragment and its immunochemical reactivity reveal that it corresponds to the NC1 domains of the α3 and α4 chains. These results reveal that the 60K component contains the NC1 domain of the α3 and α4 chains, and, therefore, probably represents two components that copurify, and that it also contains a collagenous domain.

Conclusive evidence for the existence of a collagenous domain within the 60K component(s) is revealed from calculations based on its amino acid composition and that of the NC1 domain (Table I). The calculations are based on 528 amino acid residues/molecule of 60K component and 249 residues/molecule of its collagenase-resistant fragment(s), as determined by SDS-PAGE (9) under reducing

conditions. The collagenous domain (279 residues) represents the difference between the 60K component and the NC1 domain, and as presented in Table I, its composition is characteristic of that of triple-helical collagen.

In conclusion, these results show that the subunits of the NC1 hexamer of collagen IV, identified as α3(IV)NC1 and α4(IV)NC1, exist as part of a 60K collagenous component in GBM. This provides evidence for the collagenous nature of the α3 and α4 chains. The 60K component likely represents a proteolytic fragment of yet a larger precursor component because several components exist in the GBM extract with sizes greater than 60K.

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