Quaternary Organization of the Goodpasture Autoantigen, the \( \alpha_3(IV) \) Collagen Chain

SEQUESTRATION OF TWO CRYPTIC AUTOEPITOPES BY INTRAPROTOMER INTERACTIONS WITH THE \( \alpha_4 \) AND \( \alpha_5 \) NC1 DOMAINS*

Received for publication, July 31, 2002, and in revised form, August 19, 2002.
Published, JBC Papers in Press, August 21, 2002, DOI 10.1074/jbc.M207769200

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Goodpasture’s (GP) disease is caused by autoantibodies that target the \( \alpha_3(IV) \) collagen chain in the glomerular basement membrane (GBM). Goodpasture autoantibodies bind two conformational epitopes (\( E_A \) and \( E_B \)) located within the non-collagenous (NC1) domain of this chain, which are sequestered within the NC1 hexamer of the type IV collagen network containing the \( \alpha_3(IV) \), \( \alpha_4(IV) \), and \( \alpha_5(IV) \) chains. In this study, the quaternary organization of these chains and the molecular basis for the sequestration of the epitopes were investigated. This was accomplished by physicochemical and immunochemical characterization of the NC1 hexamers using chain-specific antibodies. The hexamers were found to have a molecular composition of \( \alpha_3\alpha_4\alpha_5 \) and to contain cross-linked \( \alpha_3\alpha_5 \) heterodimers and \( \alpha_4\alpha_4 \) homodimers. Together with association studies of individual NC1 domains, these findings indicate that the \( \alpha_3 \), \( \alpha_4 \), and \( \alpha_5 \) chains occur together in the same triple-helical protomer. In the GBM, this protomer dimerizes through NC1-to-NC1 domain interactions such that the \( \alpha_3 \), \( \alpha_4 \), and \( \alpha_5 \) chains of one protomer connect with the \( \alpha_4 \), \( \alpha_5 \), and \( \alpha_3 \) chains of the opposite protomer, respectively. The immunodominant Goodpasture autoepitope, located within the \( E_B \) region, is sequestered within the \( \alpha_3\alpha_4\alpha_5 \) protomer near the triple-helical junction, at the interface between the \( \alpha_3\)NC1 and \( \alpha_5\)NC1 domains, whereas the \( E_A \) epitope is sequestered at the interface between the \( \alpha_3\)NC1 and \( \alpha_4\)NC1 domains. The results also reveal the network distribution of the six chains of collagen IV in the renal glomerulus and provide a molecular explanation for the absence of the \( \alpha_3 \), \( \alpha_4 \), \( \alpha_5 \), and \( \alpha_6 \) chains in Alport syndrome.

The glomerular basement membrane (GBM)\(^1\) is a key component of the kidney ultrafiltration barrier. Type IV collagen, the main structural component of the GBM, has been implicated in several glomerular diseases. Goodpasture’s (GP) disease is caused by anti-GBM autoantibodies that bind to the type IV collagen networks of the GBM, and, in some patients, of the alveolar basement membranes (1). The bound antibodies induce an inflammatory response causing rapidly progressive glomerulonephritis and pulmonary hemorrhage. The GP autoantibodies are specifically targeted to the non-collagenous domain (NC1) of the \( \alpha_3(IV) \) chain, the “Goodpasture autoantigen,” one of the six chains that comprise the collagen IV family (2). Two conformational GP epitopes, designated \( E_A \) and \( E_B \), have been localized within the \( \alpha_3(IV) \) NC1 domain at residues 17–31 and 127–141, respectively (3). The GP epitopes are sequestered in the type IV collagen network of native basement membranes by quaternary interactions among NC1 domains (4).

How the \( \alpha_3(IV) \) chain is organized in the collagen IV network of GBM is unknown. Collagen IV networks are composed of triple-helical protomers that are connected at the carboxyl termini by NC1-to-NC1 interactions, forming dimers of protomers, and at the amino termini by interactions involving the 7 S domain, forming tetramers (5, 6). In the GBM, an \( \alpha_1\alpha_2(IV) \) network and a distinct \( \alpha_3\alpha_4\alpha_5(IV) \) network have been identified based on differential solubilization with pseudolysin (7) and analysis of collagenase-solubilized NC1 hexamers (8). The protomer and network organization of \( \alpha_1(IV) \) and \( \alpha_2(IV) \) chains has been well established and confirmed by the recent determination of the crystal structure of the \([\alpha_1]_2\[\alpha_2]_2(IV) \) NC1 hexamer (9, 10). In contrast, little is known about the organization of \( \alpha_3(IV) \), \( \alpha_4(IV) \), and \( \alpha_5(IV) \) chains, i.e. which combinations of three chains form protomers, what is the relative position of chains within protomers, and which kinds of protomers associate through NC1-to-NC1 interactions. This information is essential for understanding the molecular basis of GP disease.

The GP epitopes are sequestered within the NC1 hexamer structure of the \( \alpha_3\alpha_4\alpha_5(IV) \) network and inaccessible for autoantibody binding unless the hexamer dissociates (4, 11). Unmasking the GP epitopes is thought to be critical for etiology and pathogenesis of GP disease, but the molecular basis for the epitope sequestration is not known (12). Based on the identification of hydrophobic residues in the epitope of the immunodominant GP\(_A\) autoantibodies, it has been proposed that the epitope is buried at the interface between interacting NC1 sequences; \( r \)-recombinant; \( E_A \) and \( E_B \), the \( \alpha_3(IV)\)NC1 residues 17–31 and 127–141, respectively, that encompass the epitopes of human GP autoantibodies; mAb, monoclonal antibody.

* This work was supported by Postdoctoral Fellowship 9920539Z from the American Heart Association-Kansas Affiliate (to D. B. H.); Grants R01 DK63925 (to M. S.) from the National Institutes of Health; and by Grant-in-aid for Scientific Research (B) 14370434 from the Japan Society for the Promotion of Science (to Y. N.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations are used: GBM, glomerular basement membrane; ELISA, enzyme-linked immunosorbent assay; GP, Goodpasture; IgG, immunoglobulin G; NC1, the noncollagenous domain of type IV collagen; \( r \)-recombinant; \( E_A \) and \( E_B \), the \( \alpha_3(IV)\)NC1 residues 17–31 and 127–141, respectively, that encompass the epitopes of human GP autoantibodies; mAb, monoclonal antibody.

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domains (13). An analysis of the homologous α1α2(IV) NC1 hexamer structure revealed that the GP epitopes are located adjacent to the triple-helical domain and to the interfaces between NC1 monomers within a protomer (9, 10).

In the present study, the quaternary organization of the α3(IV) chain in relation to the α2(IV) and α5(IV) chains in the network was investigated to determine: (a) how chains coexist in the relative disposition in the triple-helical protomer, (b) how the protomers are connected through the NC1 domains, and (c) how the GP epitopes are sequestered. The results unambiguously identify a single quaternary organization of the α2α4α5 NC1 hexamer revealing that the α3, α4, and α5 chains coexist in a single protomer that self-associates through NC1-NC1 domain interactions to form a network. Moreover, the immunodominant GP epitope, located within the E3 region, is sequestered at the α3-α5 NC1 interface within the protomer, which partially buries residues required for autoantibody binding, whereas the E6 epitope is sequestered at the α3-α4 NC1 interface.

Based on these findings, we propose that the designation of Goodpasture autoantigen should henceforth refer to the α3α4α5(IV) triple-helical protomer, rather than to the α3(IV) collagen chain, as this molecule reflects both the organization and the cryptic property of the autoantigen in the GBM.

EXPERIMENTAL PROCEDURES

**Proteins**—Human glomeruli isolated from human kidneys by differential sieving were used to purify human GBM, which was solubilized by digestion with bacterial collag enase (Calbiochem), and then fractionated by ion-exchange and gel filtration to purify the NC1 hexamers of type IV collagen (14). Recombinant (r-) human α3, α4, and α5 NC1 domains with NH2-terminal FLAG epitope were expressed in human 293 cells and purified by affinity chromatography using immobilized anti-FLAG M2 antibody (Sigma), as described previously (15). The recombinant NC1 domains were previously shown to be correctly folded and able to interact with each other (8).

**Monoclonal Antibodies and Immunoinnouitiffation Fractionation of NC1 Hexamers**—For detection of human α1–α6 NC1 domains in Western blot and ELISA, as well as for immunoprecipitation of dissociated NC1 hexamers (see below), rat monoclonal antibodies (mAbs) H11 (to α1), H22 (to α2), H31 (to α3), H43 (to α4), H52 (to α5), and H63 (to α6), previously described (16), were used, along with mouse Mab1 (to α1), Mab5 (to α3), and Mab5 (to α5), which were purchased from Wieslab AB (Lund, Sweden). A new monoclonal antibody specific for the E3 region of α3 NC1 domain, designated mAb EB3, was produced by immunizing mice with human r-α3 NC1 monomers. Its specificity was determined by ELISA against a panel of α1/α3 NC1 chimeras (3); mAb EB3 bound only the C6 chimera that contained the E3 region (residues 127–141) of α3 NC1 within an α1 NC1 scaffold. Several properties of mAb EB3 resemble those of human GP α autoantibodies: (a) its epitope is encompassed by the E3 region, (b) it is cryptic in the NC1 hexamer, and (c) it is conformational. A full description of this antibody will be presented elsewhere.

Several NC1 hexamer-binding mAbs were used for the immunoinnouitiffation fractionation of native NC1 hexamers from human GBM. Mab1 and Mab5 were used for immunoprecipitation of α1- and α3-containing native NC1 hexamers, and immobilized rat mAbs B51 (to α5) and B66 (to α6) were used for affinity chromatography of hexamers containing the α5 and α6 chains, respectively, as described (17). In addition, several novel hexamer-binding mAbs specific for the α4 NC1 domain were produced by the rat lymph node method (18) in Wistar-Kyoto rats immunized with either human r-α4 NC1 domain (mAbs RH42 and RH45) or with total human GBM hexamers (mAb N42). The specificities of these mAbs were determined by ELISA using human NC1 domains and all were found to be specific for human r-α4 NC1 monomers. Moreover, in indirect immunofluorescence of human kidney sections, mAbs RH42, RH45, and N42 exhibited a glomerular staining pattern identical to that of affinity-purified mAb H43. However, whereas mAb H43 recognized cryptic epitopes and required pretreatment of tissue sections with acid urea, the newly produced mAbs stained native sections, indicating that they bind to epitopes exposed in the NC1 hexamer. For further use in the affinity fractionation of NC1 hexamers, RH45 IgG was purified on protein G-Sepharose (Amersham Biosciences) and immobilized to Affi-Gel-10 (Bio-Rad), as described for mAbs B51 and B66 (17).

**Enzyme Immunoassays**—Affinity-purified α3α4α5(IV) NC1 hexamers, eluted from either the RH45 or the B51 affinity columns, were coated at 75–150 ng/well in microtiter plates in 3 mM guanidine chloride. Their reactivity with mAbs EB3, RH45, and H52 was measured by ELISA, using anti-rat IgG conjugated with alkaline phosphatase as secondary antibody. The amounts of α3, α4, and α5(IV) NC1 domains in the purified hexamers were quantified relative to standard curves, constructed by coating 10–100 ng of human r-α3, r-α4, and r-α5 NC1 monomers under the same conditions.

The interaction of α3 with α4 and α5(IV) NC1 monomers, in the presence of several anti-α3 antibodies (mAbs H31, EB3, and Mab3; affinity-purified (3) human GP α autoantibodies), was studied by ELISA. The r-α3, r-α4, and r-α5 NC1 monomers were immobilized overnight onto microtiter plates (500 ng/well in 50 μl) in 50 mM carbonate buffer, pH 9.6. Binding to immobilized NC1 monomers of anti-α3 NC1 antibodies, alone or preincubated with r-α5 NC1 monomers (0.5 μg/ml for 1 h at room temperature), was measured by ELISA using the appropriate secondary antibodies (anti-mouse IgG, anti-rat IgG, or anti-human IgG) conjugated with alkaline phosphatase. The measurements were performed in triplicates.

**Analysis of the NC1 Dimers of Human GBM by Immunoprecipitation**—Human GBM NC1 hexamers (2–10 μg) were dissociated in various experimental conditions and immunoprecipitated with specific antibodies against α1–α5 NC1 domains (10–20 μg). The NC1 domains bound to the precipitating antibodies were collected on protein G-Sepharose, separated by SDS-PAGE in replicate gels, and analyzed by Western blots for the presence of α1–α5 NC1 domains. The choice of dissociation conditions was important, because complete dissociation of the NC1 hexamers into subunits had to be achieved without destroying the epitopes, to allow subsequent binding of the precipitating antibodies to the NC1 monomers and dimers. The optimal conditions were empirically determined to be heating with 0.5% SDS for 10 min at 60°C, followed by a 10–20-fold dilution with Triton X-buffer (1% mg/ml bovine serum albumin). The small amount of SDS remaining did not interfere with the binding of anti-NC1 antibodies, but was sufficient to prevent further non-covalent interactions among the dissociated NC1 domains, as demonstrated by the identification of a single species of NC1 monomers in the immunoprecipitate.

**Electrophoresis and Western Blot Analysis**—PAGE was performed in gradient gels (10–22%) under non-reducing condition, using 200–300 ng of NC1 hexamer per lane. For two-dimensional polyacrylamide gel electrophoresis (two-dimensional PAGE), the NC1 hexamers (3–15 μg) were separated in the first dimension by non-equilibrium pH gradient electrophoresis (19), then in the second dimension by SDS-PAGE using a 7.5% gel gradient. The gels were silver-stained or transferred to nitrocellulose for Western blot analysis. After blocking with 2% casein, the membranes were reacted with monoclonal antibodies to α1–α5 NC1 domains (diluted 1:500–1:1000), incubated with the appropriate alkaline phosphate-conjugated secondary antibody (diluted 1:2000), then developed with 4-bromo-5-chloro-3-indolyl phosphate and nitro blue tetrazolium.

**Molecular Graphs**—The structures of the α1α2(IV), NC1 hexamers from lens capsule (Protein Data Bank ID 1M3D) (9) and placenta (Protein Data Bank ID 1LI1) (10) basement membranes were rendered using the molecular graphics visualization software RasTop (version 2.0) and analyzed using the WHAT IF web server interface (www.cmbi.kun.nl/psi/servers/WIWIFW/).

RESULTS

**Triple Helical and Network Organization of the α3(IV) Chain, the Goodpasture Autoantigen—**In the type IV collagen substructure, two triple-helical protomers interact at the carboxyl terminus via their NC1 domains, forming a stable NC1 hexamer complex that can be excised from basement membranes by collagenase digestion. Because of the special position of NC1 hexamers, connecting two adjoining protomers, the identity of monomer and dimer subunits of the hexamers reflects the chain composition of protomers, and the dimer subunits identify which chains are connected by covalent interactions between protomers. A strategy perfected for the analysis of NC1 hexamers from smooth muscle basement mem-
branes that led to the discovery of a novel α1α2α5α5α6(IV) network (17) was used here to analyze the collagen IV network containing the α3, α4, and α5 chains.

Previous studies of the NC1 hexamers from the GBM using immunoprecipitation with Mab3 antibody (to α3 NC1) revealed that α3 NC1 interacts with α4 NC1 and α5 NC1 forming a subset of NC1 hexamers distinct from the α1-α2 NC1 hexamers (8). Whether this hexamer population consisted of α3α4α5 or α3α5α5 or α3α4α5α5 heterohexamers could not be determined because only an α3-precipitating antibody was available. Recently, we developed precipitation mAbs for α5 and α6 NC1 domains, designated B51 and B66, respectively (17). Here, we introduce a novel precipitating Mab specific for the α4 NC1 domain, RH45. The availability of hexamer-binding mAbs to α3-α6 NC1 domains provided the experimental strategy to delineate hexamer organization (Fig. 1).

The hexamer population from a preparation of human glomeruli contains all six chains of type IV collagen (Fig. 1, i). Immunohistochemical studies have established that the GBM contains the α1-α5(IV) chains, Bowman’s capsule basement membrane contains the α1, α2, α5 and α6(IV) chains, and the mesangial matrix comprises the α1 and α2(IV) chains (16, 20). Hence, hexamers containing α3 and α4 (along with α5) originate exclusively from the GBM, whereas those containing α6 originate exclusively from Bowman’s capsule basement membrane. The α6-containing hexamers were removed from the mixture by adsorption to an anti-α6 affinity column (mAb B66). The B66-bound fraction, representing ~1% of the total hexamers, contained the α6 NC1 domain along with the α1, α2, and α5 NC1 domains (Fig. 1, ii). This composition, together with the mobility of dimers, indicate that the B66-bound hexamer population is identical to that identified in the smooth muscle basement membranes of aorta and bladder (17). Thus, the Bowman capsule contains the same α1α2α5α6(IV) network found in smooth muscle.

The unbound hexamers, containing the α1-α5 NC1 domains (Fig. 1, iii), were further analyzed with Mab3 (anti-α3), Mab RH45 (anti-α4), and Mab B51 (anti-α5). Mab3 bound hexamers that contained only the α3, α4, and α5 NC1 domains (Fig. 1, iv), as described previously, whereas those not bound contained only α1 and α2 NC1 domains (Fig. 1, vii). The later result indicates the presence of α1α2α(IV) hexamers (as confirmed by coprecipitation of α1 and α2 NC1 domains by Mab1; data not shown). The absence of α4 and α5 NC1 domains from the Mab3-unbound indicates that the α4(IV) and α5(IV) chains must always accompany the α3(IV) chain; this rules out the existence of α4 and α5 homohexamers as well as α4α5 heterohexamers. Likewise, Mab RH45 bound hexamers consisting of the α3, α4, and α5 NC1 domains (Fig. 1, v), whereas the unbound hexamers contained only α1 and α2 NC1 domains (Fig. 1, vii). This indicates that α3(IV) and α5(IV) chains must accompany the α4(IV) chain, further ruling out α3 and α5 homohexamers, as well as α3α5 heterohexamers. Finally, Mab B51 (Fig. 1, vi) bound hexamers containing α3, α4, and α5 NC1 domains, while the unbound fraction contained only α1 and α2 NC1 (Fig. 1, viii). Absence of the α3 and α4 NC1 domains from the unbound fraction indicates that the α3(IV) and α4(IV) chains must accompany the α5(IV) chain; this excludes α3 and α4 homohexamers as well as α3α4 heterohexamers. Together, these data indicate that the α3, α4, and α5 NC1 domains originating from the GBM exist as heterohexamers composed of all three domains, but not as homohexamers, nor as α3α4, α3α5, or α4α5 heterohexamers.

The α3α4α5 heterohexamer can theoretically exist as ten distinct isoforms that differ in the stoichiometric composition of the three NC1 domains. Examples compositions are: (α3)3(α4)2(α5)2, (α3)3(α4)3(α5)2, and (α3)3(α4)1(α5)3. To distinguish among these, the relative abundance of each NC1 domain was measured by ELISA using chain-specific monoclonal antibodies: EB3 for α3NC1, RH45 for α4NC1, and H52 for α5NC1. These antibodies yielded linear calibration curves using known amounts of recombinant human NC1 domains (data not shown). The α3, α4, and α5 NC1 domains were found in approximately equimolar proportions: 37.4% ± 6.0%, 33.7% ± 6.0%, and 28.9 ± 7.8%, respectively (means and S.D. values from five determinations). These results indicate that the hexamer has a composition of (α3)3(α4)2(α5)2.

This composition could reflect 18 distinct combinations in which two protomers (trimers of chains), varying in both chain composition and relative position of chains, interact in different trimer-trimer orientations forming a cross-linked hexamer (Fig. 2). For example, in group I all protomers contain an α3, α4, and α5 chain, but the combinations differ with respect to the trimer-trimer orientations (as in A1, A2, and A3) and with respect to the orientation of the three chains in the protomer (as in A1, B1, and C1), for a total of nine combinations. In group II, two different protomers, differing in chain composition and different from group I, associate forming three additional com-
and/H9251 for instance an (other combinations. These are:
A1, B1, C1), or the mutual orientation of the two interacting protomers (e.g. A1, B1, C1), or the mutual orientation of the two interacting protomers (e.g. A1, A2, A3). The interactions of two dissimilar protomers yields nine distinct isoforms that differ in the relative orientation of the chains within each protomer (e.g. A1, B1, C1), or the mutual orientation of the two interacting protomers (e.g. A1, A2, A3). The interactions of two dissimilar protomers yields nine additional isoforms; for each of the three combination of protomers (D-F) there are three isoforms that differ in the mutual protomer orientation (e.g. D1, D2, D3), for a total of 18 possible (a3a4a5), (a3a4a6), and (a3a5a6) isoforms.

Group I.

\[
\begin{align*}
\text{A} & : (\alpha_3\alpha_4\alpha_5) + (\alpha_3\alpha_4\alpha_5) \\
\text{B} & : (\alpha_3\alpha_4\alpha_5) + (\alpha_5\alpha_5\alpha_4) \\
\text{C} & : (\alpha_3\alpha_4\alpha_5) + (\alpha_3\alpha_4\alpha_5) \\
\text{D} & : (\alpha_3\alpha_4\alpha_5) + (\alpha_3\alpha_4\alpha_5) \\
\text{E} & : (\alpha_3\alpha_4\alpha_5) + (\alpha_3\alpha_4\alpha_5) \\
\text{F} & : (\alpha_3\alpha_4\alpha_5) + (\alpha_3\alpha_4\alpha_5)
\end{align*}
\]

**Fig. 2.** Theoretical isoforms of an NC1 hexamer with an (a3a4a5)/(a3a4a5) composition. An (a3a4a5)/(a3a4a5) NC1 hexamer could be assembled either from two a3a4a5 protomers interacting with each other (A-C), or from the association of two dissimilar protomers (D-F), for instance an (a3a4a5) protomer interacting with an (a3a5a6) protomer. Interaction of two a3a4a5 protomers yields nine distinct isoforms that differ in the relative orientation of the chains within each protomer (e.g. A1, B1, C1), or the mutual orientation of the two interacting protomers (e.g. A1, A2, A3). The interactions of two dissimilar protomers yields nine additional isoforms; for each of the three combination of protomers (D-F) there are three isoforms that differ in the mutual protomer orientation (e.g. D1, D2, D3), for a total of 18 possible (a3a4a5)/(a3a4a5) isoforms.

**Analysis of Interprotomer Interactions**—Many of these combinations can be excluded by the identification of NC1 monomers that are cross-linked forming dimers. In most basement membranes, including the GBM, the NC1 hexamer contains a large proportion of NC1 dimers (~75%) cross-linked by a covalent bond that connects two adjoining protomers in the network. Previously, this cross-link was identified as a disulfide bond (21), but recent crystallographic studies suggested a cross-link between the conserved Met-93 and Lys-211 residues (10). Because each NC1 domain of a protomer can be cross-linked to the corresponding NC1 domain of the opposite protomer, each hexamer is characterized by three NC1 dimers that reflect specific trimer-trimer interactions. Thus, the identity of dimers that exist in the a3a4a5 heterohexamer reflects which chains are connected between protomers. Overall, the 18 hexamer isoforms shown in Fig. 2 contain six possible dimer combinations. These are: a3a3, a3a4, and a3a5 NC1 homodimers and a3a4, a3a5, and a4a5 NC1 heterodimers. The absence of some of these NC1 dimer combinations would rule out the existence of certain hexamers isoforms. Two independent methods were used for this determination.

**Fig. 3.** Analysis of the NC1 dimers from human GBM by twodimensional gel electrophoresis. The NC1 hexamers from the GBM (3–15 µg) were separated by two-dimensional PAGE and either silver-stained for total protein (S) or transferred to nitrocellulose for Western blot with chain-specific antibodies, as indicated (a1–a6). Under these conditions, the NC1 hexamers are resolved into their component subunits, NC1 monomers (m) and covalently linked dimers (d). The assignment of dimer spots was based on the pattern of staining with chain-specific mAbs. Migration of NC1 dimers to a unique characteristic position, distinct from all other NC1 domains, indicates the existence of homodimers (a1-a1, a2-a2, and a4-a4). The overlap between the a3 and a5 NC1 dimers is strongly suggestive of a3-a5 heterodimers, but a3-a3 or a3-a5 homodimers could not be ruled out.

Under the separation conditions in two-dimensional gel electrophoresis, the NC1 hexamers dissociate into their component subunits, NC1 monomers and dimers, which migrate to distinct positions according to their size and isoelectric point (22, 23). Following separation by two-dimensional electrophoresis and transfer to nitrocellulose, the NC1 domains from human GBM hexamers were identified by Western blot with chain-specific mAbs (Fig. 3), and the migration position of the NC1 dimers was analyzed. Both a1 and a2 NC1 dimers migrated to characteristic positions indicative of a1-a1 and a2-a2 homodimers, consistent with the known organization of the a1-a2 (IV) hexamer, in which the two (a1),a2 protomers are cross-linked by a1-a1 and a2-a2 bonds. Among the a3, a4, and a5 NC1 domains, only a4 NC1 dimers migrated to a distinct position, which demonstrates the existence of a4-a4 homodimers but rules out a4-a3 and a4-a5 heterodimers. The positions of a3 and a5 NC1 dimers overlapped significantly, suggesting the possible existence of a3-a5 heterodimers, as suggested previously (24).

However, because comigration does not prove the existence of a covalent link, the NC1 dimers of human GBM were also analyzed by immunoprecipitation followed by SDS-PAGE analysis (Fig. 4). This was achieved by first dissociating the NC1 hexamers into their component subunits (NC1 monomers and dimers), followed by precipitation with chain-specific antibodies (Fig. 4, top). Dissociation conditions were chosen that achieved complete dissociation of the hexamer, while allowing subsequent binding of the antibodies to the NC1 monomers and dimers subunits. Upon reaction with dissociated GBM
The existence of α3-α4 homodimers and α3-α5 heterodimers, along with the absence of α3-α4 and α4-α5 heterodimers, excludes 14 out of 18 possible α3-α4, α5(IV) hexamer isoforms. Among the four remaining isoforms, three (designated “A1,” “B1,” and “C1” in Fig. 2) represent the association of two protomers, each containing all three chains, but which differ in the relative position of the chains. Of these, isoform C1 does not contain an α3-α5 heterodimer linkage; thus, while its existence cannot be excluded, it must coexist with another isoform(s). The fourth one (designated as “D1” in Fig. 2) represents an (α3)2,α4 protomer associated with an (α5)2,α4 protomer. Isoform D1 is unlikely because it is not consistent with the results of in vitro reassembly among α3, α4, and α5 NC1 monomers (8). Those studies showed that α3 NC1 monomers could form binary complexes with both α4 and α5 NC1 monomers, but α4 and α5 NC1 monomers did not associate with each other in the absence of α3 NC1.

Analysis of Interprotomer Interactions—Further discrimination among the remaining possibilities required an analysis of the intraprotomer interactions among α3, α4, and α5 NC1 domains. The recently solved crystal structure of the [α3(α1)2]3 NC1 hexamer (9, 10) provides a framework for this analysis. Due to the high sequence homology among the six NC1 domains (52–83% identity), the α3-α4-α5 NC1 hexamer must have a similar tertiary and quaternary structure. The NC1 hexamer is formed of two identical trimeric caps, each derived from one protomer, that interact through a large planar surface (one trimeric cap is shown in Fig. 5a). Within each trimeric cap, three NC1 domains (shown in red, green, and blue) are arranged radially around a pseudo-3-fold axis (Fig. 5b). Each NC1 domain is composed of two homologous subdomains, an amino-terminal subdomain A (lighter shade) and a carboxyl-terminal subdomain B (darker shade). As illustrated for the α3 NC1 domain, within the NC1 trimer, subdomain A of each monomer interacts with subdomain B of the next monomer. Thus, an α3 NC1 monomer has two distinct neighbors, “x” and “y,” interacting with its subdomains A and B, respectively. The identity of x and y distinguishes among the four remaining possible isoforms of the α3-α4-α5 hexamer actually exist (Fig. 5c).

Their identities were determined using antibodies targeted to different regions of the α3 NC1 domain, which block the interactions of either x or y with the α3 interface. Our previous studies established that recombinant human α3, α4, α5 NC1 monomers associate with each other in vitro and that all three are required to form an NC1 hexamer (8). Here, we showed that several antibodies to the α3 NC1 domain could selectively block the interaction of α3 NC1 with either α4 or α5 NC1 domains, but not both. Because the epitopes of these antibodies are known and they are located in the proximity of either x or y NC1 domains (Fig. 6, top), this information could be used to identify x and y and, therefore, the relative orientation of α3, α4, and α5 chains within the protomer. GPA auto-Abs bind to the Ea region (residues 17–31) (3), located proximal to the x, but not y, monomers. mAb EB3 specifically recognizes the Ep region (residues 127–141), and the epitope of mAb H31 was mapped to residues 208–214 (IPSTVKA) (18), both located proximal to y, but not x, monomers. The epitope of Mab3 is jointly formed by the Ea and Ep regions (4). Binding of these antibodies to the α3 NC1 monomer would either allow or prevent the interactions of the α3 NC1 with the α4 and/or α5 NC1 monomers.

The ability of anti-α3 NC1 antibodies to block the interaction between soluble α3 NC1 monomers and immobilized α4 and α5 NC1 monomers was studied by ELISA, using the appropriate secondary antibodies to detect the formation of a trimolecular complex. Control experiments showed that in the absence of α3 NC1, anti-α3 antibodies did not react with immobilized α4 or α5 monomers (Fig. 6, solid bars), indicating that when an α3 NC1-antibody complex bound to the immobilized α4 and/or α5 NC1 domains (Fig. 6, gray bars), the interaction was mediated by the α3 NC1 domain. When in complex with Mab3, the α3 NC1 domain could interact with both α4 and α5 NC1 monomers...
Follows that of these interactions, which is consistent with the accessibility bodies and x

Because their epitopes are proximal to the structures of the monomers can form a binary complex with either NC1 domain, but not with the NC1 domain, it follows that NC1 domain, whereas NC1 remains to be determined. The NC1 domains interacting with subdomains A and B of the NC1 hexamer were designated x and y, respectively. The four possible isoforms of the NC1 hexamer differ in the identities of x and y; the prime symbol was used to distinguish between the three NC1 monomers (0.5 g/ml), was measured using appropriate secondary antibodies (gray bars). In control experiments, anti-α3 antibodies alone did not interact with α4 or α5 NC1 domains (solid bars).

In complex with Mab3, α3NC1 interacted with both α4 and α5 NC1 monomers. In complex with GPA auto-Abs, α3NC1 interacted with α4 but not α5 NC1 monomers. In complex with EB3 or H31, α3NC1 interacted with α5 but not α4 NC1 monomers. Together, these results indicate that x is the α5 NC1 domain, and y is the α4 NC1 domain.

**DISCUSSION**

**Organization of Chains within the α3α4α5(IV) Network**—The NC1 domains play a critical role at multiple stages in the assembly of collagen IV networks. First, interactions among NC1 domains initiated assembly of three chains into a triple-helical protomer (26). Second, NC1 domains mediate the association of two protomers head-to-head, forming at the junction an NC1 hexamer, which in turn is stabilized by cross-links (5). Third, the NC1 domains encode the specificity of interactions among the α1–α6(IV) chains at two levels: (a) the selection of

(Fig. 6a). This confirms the previous observation that α3 NC1 monomers can form a binary complex with either α4 and α5 NC1 monomers (8). Furthermore, Mab3 does not hinder either of these interactions, which is consistent with the accessibility of Mab3 epitope in the α3α4α5 hexamer (4, 8, 25).

In contrast to Mab3, the complex between GPA autoantibodies and α3 NC1 monomers could interact with the α4, but not with the α5 NC1 monomers (Fig. 6b). Thus, GPA antibodies block specifically the interaction of α3 NC1 with α5 NC1, but not with α4 NC1. Because their epitope, the E4 region of α3 NC1, is proximal to the x, but not to y, NC1 domain, it follows that α5 is not allowed at position y and hence must occupy position y, whereas α5 is allowed at position x. This further rules out α3α4α5(IV) isoforms B1 and C1 (Fig. 5), leaving only one possible isoform, A1. Together, these data indicate that the α3, α4, and α5 chains are arranged in the protomer such that subdomain A of the α3 NC1 domain interfaces with subdomain B of the α5 NC1 domain, whereas subdomain B of α3 NC1 interfaces with subdomain A of the α4 NC1 domain.

**Fig. 5. Organization of the NC1 hexamers.** The x-ray crystal structures of the [α1α2] NC1 hexamers from the lens capsule (9) and placenta (10) basement membranes reveal that the NC1 hexamers are composed of two trimeric caps, each derived from one protomer, interacting through a planar interface (arrow). A lateral view (a) and a view from the trimer-trimer interface (b) show that within each trimeric cap, the three NC1 domains (shown in red, green, and blue, respectively) are arranged radially such that subdomain A (lighter color) of one chain interacts with subdomain B (darker color) of the next chain. The high homology between α1–α6 chains indicates that the α3α4α5 NC1 hexamer must have tertiary and quaternary structures similar to the [α1α2] NC1 hexamer, but the relative positions of the α3, α4, and α5 NC1 remain to be determined. NC1 domains interacting with subdomains A and B of the α3NC1 were designated x and y, respectively. The four possible isoforms of the α3α4α5 NC1 hexamer differ in the identities of x and y; the prime symbol was used to distinguish between the two α3 NC1 monomers of the α3α4α5 hexamers (c).

**Fig. 6. Anti-α3 NC1 antibodies selectively block binding of the α3 NC1 monomers to either α4 NC1 and α5 NC1 monomers.** The epitopes of several anti-α3 antibodies are located in the proximity of either x or y NC1 domains that interact with an α3 NC1 domain (top). The ability of these antibodies to block the interaction of α3 with α4 or α5 NC1 domains was measured by ELISA. The r-α3, r-α4, and r-α5 NC1 monomers were immobilized overnight onto microtiter plates (500 ng/well in 50 μl of carbonate buffer). Binding of anti-α3 NC1 antibodies Mab3 (a), GPα autoAbs (b), mAb EB3 (c), and mAb H31 (d), in complex with r-α3 NC1 monomers (0.5 μg/ml), was measured using appropriate secondary antibodies (gray bars). In control experiments, anti-α3 antibodies alone did not interact with α4 or α5 NC1 domains (solid bars). In complex with Mab3, α3NC1 interacted with both α4 and α5 NC1 monomers. In complex with GPA auto-Abs, α3NC1 interacted with α4 but not α5 NC1 monomers. In complex with EB3 or H31, α3NC1 interacted with α5 but not α4 NC1 monomers. Together, these results indicate that x is the α5 NC1 domain, and y is the α4 NC1 domain.

Goodpasture Autoantigen of the Glomerular Basement Membrane
chains for protomer assembly and the relative orientation of the three chains within the protomer and (b) the selection and relative orientation of the two adjoining protomers (8, 17). Thus, the quaternary structure of the NC1 hexamer reflects the molecular interactions governing the affinity, organization, and specificity of network assembly. Previous studies of NC1 hexamers have revealed the existence of three distinct collagen IV networks: an ubiquitous one containing the a1 and a2(IV) chains; another containing the a3, a4 and a5(IV) chains, found in the GBM, and other containing the a1, a2, a5, and a6(IV) chains, found in the smooth muscle basement membranes. The organization of chains at the protomer and network level has been determined for the a1-a2(IV) and a1-a2-a5-a6(IV) networks, but not for the a3-a4-a5(IV) network.

In the present study, the chain organization of the a3-a4-a5(IV) network of the GBM was determined to define the quaternary organization of the GP autoantigen, the a3(IV) chain. This was accomplished using a combination of techniques, including immunochemical analysis of native and dissociated NC1 hexamers, two-dimensional electrophoresis, and analysis of the specificity of interactions between NC1 monomers. The results establish that the a3(IV) chain, along with an a4(IV) and an a5(IV) chain, exist as a single triple-helical protomer, which self-associates forming a dimer through NC1-to-NC1 interactions (Fig. 7A). At the protomer level, the amino-terminal subdomain A of the a3 NC1 domain interfaces with the carboxyl-terminal subdomain B of the a5 NC1 domain, and its carboxyl-terminal subdomain B interfaces with the N-terminal subdomain A of the a4 NC1 domain. At the network level, the NC1 trimer-trimer interface between two adjoining protomers is formed by interactions between the a3 NC1 domain of one protomer and the a5 NC1 domain of the other and between the a4 NC1 domains of each protomer; these connections are stabilized by cross-links.

In comparison, the a1-a2(IV) network is composed of (a1)_2 protomers (9, 10, 21), whereas the a1-a2-a5-a6(IV) network is composed of (a1)_2 protomers interacting with (a5)_2 protomers (17). Thus, the three known collagen IV networks are assembled from three basic building block protomers with the chain compositions of (a1)_2, a3-a4-a5, and a3-a5. Each protomer contains two odd-numbered chains (a1, a3, or a5) and one even-numbered chain (a2, a4, or a6), indicative of general composition of (a1-like) a2-like chain, as categorized from the sequence homology (27). At the NC1 trimer-trimer interface of the a1-a2(IV) network, the (a1)_2 protomers interact such that their respective a1 NC1 domains interact forming a1-a1 homodimers, as do their a2 NC1 domains forming a2-a2 homodimers. Likewise, in the a1-a2-a5-a6(IV) network, the (a1)_2 protomer interacts with the (a5)_2 protomer such that their respective a1 and a5 NC1 domains interact forming an a1-a5 heterodimer, and their a2 and a6 NC1 domains interact forming an a2-a6 heterodimer. In the a3-a4-a5(IV) networks, the NC1 trimer-trimer interface involves the interactions of an a3 with an a5 NC1 domain, forming an a3-a5 NC1 heterodimer, and of an a4 with another a4 NC1 domain, forming an a4-a4 homodimer. Thus, in all hexamers, the NC1 trimer-trimer interface involves contacts between a1-like chains and between a2-like chains, which are stabilized by cross-links. This feature specifies only one possible orientation at the interface, because each protomer contains only one a2-like chain.

**Distribution of Type IV Collagen Networks in the Glomerulus: Implications for Alport Syndrome**—The distribution of the six collagen IV chains in the glomerulus has been determined by immunohistochemical studies (16). The a1 and a2(IV) chains occur in the GBM, Bowman’s capsule, and mesangial matrix, whereas the a3 and a4(IV) chains occur exclusively in the GBM and the a6(IV) chain only occurs in Bowman’s capsule. In contrast, the a5(IV) chain occurs both in the GBM and Bowman’s capsule. The findings herein provide an interpreta-

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**Fig. 7.** A, quaternary organization of the (a3-a4-a5), hexamer. The building block of the a3-a4-a5(IV) network is a protomer containing one a3 chain (red), along with an a4 (blue) and an a5 (green) chains. Within the protomer, subdomain A of a3NC1 interfaces with subdomain B of a5NC1, and subdomain B of a3NC1 interfaces with subdomain A of a4NC1. Two a3-a4-a5 protomers associate further through NC1-NC1 interactions, forming an (a3)(a4)(a5), NC1 hexamer in which the a3, a4, and a5 NC1 domains of one protomer connect with the a5, a4, and a3 NC1 domains of the other protomer, respectively. The Ea (orange) and Eb (purple) regions of the a3(IV) NC1 domain, which encompass the epitopes of GPa and GPb autoantibodies, are away from the interface between protomers (interrupted yellow line), but adjacent to the a3 interfaces with the a5 and a4 NC1 domains, respectively, within the protomer. Shielding of some Ea and Eb epitope residues by a5 and a4 NC1 domains, respectively, within the protomer, is the probable cause for sequestration of the GP epitopes. B, distribution of collagen IV networks within the renal glomerulus. An a1-a2(IV) network composed of (a1)_2 protomers exists in the mesangial matrix (MM), GBM, and the basement membrane of Bowman’s capsule (BC). An a1-a2-a5-a6(IV) network, composed of (a1)_2 protomers interacting with (a5)_2 protomers, is present in Bowman’s capsule. The a3-a4-a5(IV) network, composed of a3-a4-a5 protomers, exists within the GBM. The organization of the NC1 hexamers within each of the three networks is shown in the inset.
tion of the glomerular distribution of the six chains in term of their molecular organizations in networks (Fig. 7B). An α3α4α5(IV) network composed of α3α4α5 protomers exists in the GBM. An α1α2α5α6(IV) network composed of α1α2α5 and α3α4α6 protomers exists in Bowman’s capsule. An α1α2α4(IV) network exists in the GBM, Bowman’s capsule, and mesangial matrix.

The existence and distribution of these networks provide a molecular explanation for the absence of the α3, α4, α5, and α6(IV) chains from the glomerulus in Alport syndrome, a hereditary form of progressive renal disease caused by mutations in collagen IV genes. In the X-linked form of the disease, loss of the α3 protomer altogether or result in defective protomers that are degraded or cannot self-assemble into networks, causing the loss of the α3–α6(IV) chains. In comparison, mutations in the α3(IV) or α4(IV) chains in the autosomal form of the disease cause the absence of the α3, α4, and α5(IV) chains from the GBM, but do not affect the presence of the α5 and α6(IV) chains in Bowman’s capsule (33, 34). Hence, these mutations prevent the assembly of the α3α4α5 protomer and network in the GBM, but do not affect the assembly of the α3α4α6 protomer of Bowman’s capsule.

Sequestration of Goodpasture Autoepitopes—Previous studies have established that the epitopes of GPα autoantibodies are sequestered within the NC1 hexamers and hence remain inaccessible for autoantibody binding unless the hexamer dissociates (4, 11, 12). However, the molecular basis of this cryptic property has remained unknown. Based on the hydrophobic character of the amino acids that compose the GPα epitope (Ala, Val, Ile), it has been proposed that the epitopes are buried at the interfaces between interacting NC1 domains within a hexamer (13). Determination of the x-ray structure of the homologous [α1α2α3]α4α5(IV) NC1 hexamer (9, 10) has revealed that the EA and EB regions, encompassing the epitopes of GPα and GPβ autoantibodies, are located adjacent to the triple-helical domain and to the interfaces between NC1 monomers within a protomer, but distant from the NC1 trimer-trimer interface. However, which NC1 domains sequester the GP epitopes have not been determined. Here, we showed that within the α3α4α5 protomer, the EA epitope region interfaces with the α5 NC1 domain, and the EB epitope region interfaces with the α4 NC1 domain. Thus, intraprotomer interactions of α3α5 with α5 NC1 domain appear to reduce the accessibility of certain residues within the EB epitope that are required for the binding of GPα autoantibodies (and interactions of α3NC1 with α4NC1 would likewise sequester certain residues in the EB epitope). Conversely, binding of GPβ autoantibodies to the EB epitope prevents the interaction of α3NC1 with α5 NC1, as experimentally determined.

Knowledge of the amino acids that comprise the epitope of GPα antibodies, along with the structural alignment of the α3, α4, and α5 NC1 sequences within the [α1α2α3]α4α5(IV) NC1 hexamer structure, allowed an inference about which residues contribute to the sequestration the GPα epitope. Within the EB region of the α3 NC1 chain, four amino acids (Ala-18, Ile-19, Val-27, and Pro-28) were found critical for binding of GPα autoantibodies (13), and outside this region, Gln-57 may also be required (35). A comparative analysis of the solvent-accessible surfaces of the NC1 monomers and hexamers revealed no changes in the accessibility of the Ala-18, Ile-19, and Pro-28 residues. However, the accessibility of Val-27 and Gln-57 side chains was significantly lower in the NC1 hexamer than in the monomer, indicating that partial burial of these residues likely contributes to the cryptic property of the GPα epitope. Only two other residues within the EA region, Leu-29 and Tyr-30, become buried in the NC1 hexamer and may potentially contribute to the epitope sequestration. Whether these residues are important for GPα antibody binding could not be determined in previous studies using homologue scanning mutagenesis, because Leu-29 and Tyr-30 are conserved between α1 and α3 NC1 domains. Further progress in understanding the molecular basis for the cryptic nature of the GP epitopes will be achieved by structural studies of the α3 NC1 domain in monomer form, in the NC1 hexamer complex, and in complex with (auto)antibodies against the EA or EB regions.

Acknowledgments—Human kidneys not suitable for transplantation were kindly provided by Midwest Organ Bank, Kansas City, for preparation of human GBM. Larry Howell contributed the artwork in Fig. 7B.

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