Glomerular Basement Membrane

IDENTIFICATION OF DIMERIC SUBUNITS OF THE NONCOLLAGENOUS DOMAIN (HEXAMER) OF COLLAGEN IV AND THE GOODPASTURE ANTIGEN

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The noncollagenous (NC1) domain hexamer of glomerular basement membrane (GBM) collagen is composed of a multiplicity of monomeric and dimeric subunits, and specific subunits are the targets for anti-GBM autoantibodies of patients with Goodpasture (GP) syndrome. The identity of GBM monomers has been established and the α3(IV)NC1 monomer identified as the one that binds GP antibodies (Gunwar, S., Sauss, J., Noelken, M. E., and Hudson, B. G. (1990) J. Biol. Chem. 265, 5446-5469). In the present study, the chain origin of 25 dimeric components and the identity of those that bound the anti-GBM antibodies from two GP patients were determined. This was accomplished by NH2-terminal sequence analysis and immunoblotting analysis of dimeric components that were resolved by two-dimensional electrophoresis in combination with high pressure liquid chromatography. The results revealed that (a) the components are mainly homodimers of the NC1 domains of α1, α2, α3, α4, and probably α5 chains of collagen IV, reflecting a specificity of protomer-protomer association and (b) each homodimer had several size and charge isoforms. The GP antibodies bound exclusively to both α3(IV)NC1 monomers and dimers and not to other basement membrane constituents. These findings provided new insights about the structure of GBM collagen and together with our previous findings firmly established the α3(IV) chain as the target for the anti-GBM antibodies that mediate glomerulonephritis and pulmonary hemorrhage in patients with Goodpasture syndrome.

Type IV collagen is the major protein constituent of mammalian basement membranes such as renal glomerular and lens basement membranes. This component exists as a supramolecular structure which binds other macromolecular constituents, i.e., laminin, heparan sulfate proteoglycan, and entactin (1, 2). The protomeric form of collagen IV is composed of three α chains, and it is characterized by three structural domains: 7 S at the amino terminus, triple-helical in the middle region, and NC1 at the carboxyl terminus. The classical protomer is composed of two α1 chains and one α2 chain (1, 2). Other kinds of protomers, differing in chain composition, also exist, as three other collagen IV chains (α3, α4, and α5) have now been identified (3-10).

The NC1 domain is of particular interest because of its important role in assembly of the collagen suprastructure and its involvement in immune-mediated renal disease. This domain is a critical site for cross-linking two adjoining protomers (1, 2) and for the lateral assembly of protomers (11), forming a supramolecular structure. It can be excised from the suprastructure by collagenase digestion, and under non-denaturing conditions, it exists as a hexamer whose subunits correspond to the NC1 domains of the six α chains comprising the adjoining protomers. In the presence of SDS, it dissociates into monomeric and dimeric subunits (disulfide-cross-linked monomers) (12, 13).

The NC1 domain of GBM collagen is the primary target for anti-GBM antibodies of patients with Goodpasture syndrome (3, 5, 15-17) and for anti-GBM autoantibodies of patients with Alport syndrome (18-21). In both cases, the antibodies bind to both monomer and dimer subunits. The intensity of binding is greatest to dimers, which constitute about 64% of the mass of NC1 hexamer (22). The monomers have been characterized by two dimensional electrophoresis and identified as α1NC1, α2NC1, α3NC1, α4NC1, and their isoforms (6). The α3NC1 monomer has been identified as the one that binds GP antibodies (5, 6, 15, 23). The chain origin of the dimers has not been determined, nor has the identity of those dimers that bind GP autoantibodies and Alport autoantibodies. There are 20 or more dimers (22) and this large number presumably reflects the presence of the NC1 domains of all five α chains and their respective isoforms, and the presence of homodimers and heterodimers.

The abbreviations used are: NC1, noncollagenous domain; GBM, glomerular basement membrane; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; ELISA, enzyme-linked immunosorbent assay; GP syndrome, Goodpasture syndrome; EHS, Engelbreth-Holm-Swarm.

GP syndrome is an autoimmune disorder in human beings consisting of the triad of glomerulonephritis, lung hemorrhage, and anti-GBM antibody formation (14). It includes a broad spectrum of clinical features, ranging from massive pulmonary hemorrhage with little overt evidence of renal disease to fulminant crescentic glomerulonephritis and little overt evidence of pulmonary hemorrhage.

Classical Alport syndrome is a heritable disorder in human beings that eventually causes kidney failure and deafness and is characterized by morphological abnormality in GBM (18-21). Some Alport patients develop anti-GBM autoantibodies after renal transplantation, causing loss of allograft function.
The purpose of the present study was to determine the chain origin of all dimer subunits of the GBM hexamer and to determine the identity of those that bind GP antibodies. This was accomplished by NH\textsubscript{2}-terminal sequence analysis and immunoblotting analysis of dimers that were resolved by two-dimensional electrophoresis in combination with HPLC. The results revealed that the dimers are mainly homodimers of the NC1 domain of the respective five \(\alpha\) chains, and that those comprised of \(\alpha\)NC1 bind GP antibodies. These findings provided new insights about the structure of GBM collagen and further established that the pathogenic GP antibodies are targeted to the NC1 domain of the \(\alpha\)3(IV) chain of basement membrane collagen.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine kidneys were collected as described previously (24). Bacterial collagenase (CLPSA) was obtained from Worthington Biochemicals, DE-52 cellulose from Whatman, Sephacryl S-200, S-300 from Pharmacia LKB biotechnology Inc., C\textsubscript{2}H, reverse-phase column from Vydac, keyhole limpet hemocyanin, and benzamidine HCl from Sigma, phenylmethylsulfonyl fluoride from Research Organics, Immobilon from Millipore, sequencing-grade acrylamide (Protogel) from National Diagnostics, and secondary antibody conjugates from Dako Products. Prestained protein markers were from Bethesda Research Laboratories, collagen IV from Calbiochem, fibronectin from Sigma, and heparan sulfate proteoglycan from Sigma.

**Purification of NC1 Hexamer from Bovine GBM**—GBM was prepared from bovine kidneys as previously described (24). NC1 (hexamer) domain from GBM was purified by the procedures described previously (22) under associative conditions.

**Preparation of Laminin and Entactin**—Laminin and entactin were prepared as described previously (25).

**Isolation of Dimers (D1 and D2) and Monomers of NC1 Hexamer**—NC1 hexamer (5 mg) was dialyzed against 6 M guanidine-HCl for 2 days, then heated to 85 °C for 10 min. The dissociated hexamer was fractionated on a Sephacryl S-200 column, equilibrated with 6 M guanidine HCl in 50 mM Tris-HCl, pH 7.5, to resolve dimeric and monomeric subunits. The dimer fraction was identified, pooled, concentrated, and further fractionated on a reversed-phase C\textsubscript{2}H column, equilibrated with 30% acetonitrile and 0.1% trifluoroacetic acid. The monomer fraction was resolved on the C\textsubscript{2}H column as previously described (6, 15) to obtain \(\alpha\)1NC1, \(\alpha\)2NC1, \(\alpha\)3NC1, and \(\alpha\)4NC1 monomer for ELISA experiments.

**Electrophoresis Techniques**—Two-dimensional electrophoresis was performed in the first dimension according to O'Farrel et al. (26), with minor modifications (6). In the second dimension, SDS-PAGE was performed according to Laemmli (27) using 8–22% gradient gels. Quantitative scanning of two-dimensional gels was performed at Protein Databases, Inc. (Huntington Station, NY).

**Amino Acid Sequence Analysis**—Amino acid sequence analysis was performed on proteins blotted on Immobilon after two-dimensional electrophoresis according to the procedure previously described (28, 29) with modifications as follows. Lyophilized protein (150–300 \(\mu\)g) was dissolved in 40 \(\mu\)l of 8 M urea lysis buffer and used for the first dimension; second dimension gels were cast 1 day before and equilibrated in lower gel buffer; and electrophoresis was carried out at 40 mA for 6–8 h and blotting for 14–16 h onto Immobilon in Tris-glycine buffer with 20% methanol. The Immobilon was washed for 2–3 min twice with distilled water, stained with Coomassie Blue, and destained with 50% methanol and 10% acetic acid. Then the blot was washed for 10 min with water and dried. The protein bands were excised and used directly for sequencing. Amino-terminal sequence analysis was performed at the Protein Microsequencing Facility (University of California, Los Angeles, Department of Biological Chemistry, UCLA School of Medicine).

**Immunohistological Techniques**—To prepare Western blots, the separated proteins were electrophoretically transferred to nitrocellulose, blocked with bovine serum albumin, reacted with primary antibody and secondary antibody, and stained with diaminobenzidine. Horseradish peroxidase conjugate was used as secondary antibody. Anti-GM antibodies were from the two GP patients (LL and JB) described herein. Antiserum against the \(\alpha\)1NC1 and \(\alpha\)2NC1 synthetic peptides CGGGPFGV5VDHFLVTNQSTKT and CGMPGRSIL-GYLWLK, respectively, were raised according to the procedures described previously (6). Antiserum raised against a synthetic peptide \(\alpha\)3NC1 has been described previously (6). Antiserum was raised against \(\alpha\)4NC1 peptide sequence GPGGGYGGLSSFLVLL (6). Antiserum against \(\alpha\)5(IV) peptide SDMPSKQOSG was raised according to the procedures described previously (7). All peptide antisera were further purified by affinity techniques using the respective IgG coupled to cyanogen bromide-activated matrix. The specificity of these sera, except the anti-\(\alpha\)5NC1 serum, was confirmed by immunoblots using pure antigens. Direct ELISA was performed as described earlier (16). Plates were coated with 200 ng of antigen and left overnight at room temperature. The plates were washed with 0.15 M NaCl with 0.05% Tween 20 and subsequently blocked using 1% bovine serum albumin. The plates were then incubated with the primary antibody (GP plasma or normal serum) for 1 h (1:50 dilution), washed with 0.15 M NaCl with 0.05% Tween 20, and then incubated with secondary antibody against human IgG conjugated to alkaline phosphatase (1:1000 dilution). Detection was done by addition of substrate, disodium 5-nitrophenyl phosphate (5 mg/ml), and absorbance was measured at 405 nm.

**RESULTS**

**Experimental Approach**—The NC1 hexamer was characterized to serve as reference for the identification of all dimeric components and to determine whether \(\alpha\)3NC1 monomer exists along with the \(\alpha\)3NC1 and \(\alpha\)4NC1 monomers that were previously identified (5, 6). The hexamer was characterized by two-dimensional electrophoresis, and the \(\alpha\)3NC1, \(\alpha\)4NC1,
and α5NC1 components were identified by immunoblot analysis using synthetic peptide antibodies that were directed against these NC1 domains. To facilitate their identification, dimers were first fractionated by HPLC to resolve α1NC1 and α2NC1 dimers (fraction D1) from α3NC1, α4NC1, and α5NC1 dimers (fraction D2). Dimers comprising fractions D1 and D2 were then resolved by two-dimensional electrophoresis, and each component was subsequently identified by NH2-terminal sequence analysis and by immunoblot analysis using synthetic peptide antibodies. The identity of dimeric components was prerequisite to the identification of the chain of origin of those that bound GP anti-GBM antibodies. The plasma from two GP patients was used in this study, and the plasma titer of GP antibodies in one patient was monitored over the course of therapy.

Analysis of NC1 Hexamer by Two-dimensional Electrophoresis and Immunoblots—The NC1 hexamer of bovine GBM collagen displayed a complex pattern on analysis by two-dimensional electrophoresis, showing monomeric and dimeric components (Fig. 1, panel A). The identity of monomers (panel A) was determined in previous studies (6, 22). The dimers consisted of at least 22 different components of variable staining intensities. This pattern showed more components in the pH 7–8 range and a higher quality of resolution than previously reported (22). These differences primarily reflected the use of 8 M urea in sample and gel buffer in the present study.

The identity of hexamer components was investigated using synthetic peptide antibodies that were directed to the NC1 domains of α3, α4, and α5 chains. The immunoblot pattern was distinct (panels D–F) for each antibody indicating the existence of α3NC1, α4NC1, and α5NC1 in both monomeric and dimeric forms. The identity of the α3NC1 and α4NC1 monomers was previously described. There was some overlap in dimer components that bound α3NC1 and α5NC1 antibodies, and these dimers also comigrated with dimers of α1NC1 and α2NC1 as described below. These results indicated that the dimers are comprised of α3NC1, α4NC1, and α5NC1, as well as α1NC1 and α2NC1 (vide infra).

The hexamer components were analyzed for their capacity to bind antibodies from two patients (LL and JB) with Goodpasture syndrome (panels B and C). In both cases, the antibodies bound to the same monomeric and dimeric components. The binding was specific to the α3NC1 monomers, as noted in panels B and C, as previously described (5, 6). The binding to dimers coincided with dimers that also bound α3NC1 antibody. These results suggested that GP antibodies

\(^4\) α3aNC1 and α3bNC1 are equivalent to M2*a and M2*b, respectively, and they differ by the deletion of 9 residues from the amino terminus of α3aNC1. α3aNC1 and α3bNC1 bind GP antibodies with equal intensity. Synthetic peptide antibodies were prepared against the NH2-terminal region of α3aNC1 and therefore they do not react with α3bNC1 (6).
Fig. 2. Fractionation of dimeric subunits of GBM NC1 hexamer on reversed-phase C₄ column and analysis of fractions by ELISA. The dimer fraction from a Sephacryl S-200 column (22) was further fractionated by HPLC using a C₄ reversed-phase column (panel A). Fractions D₁ and D₂ were each analyzed for their content of NC1 subunits by ELISA, using the synthetic peptide antibodies cited in Fig. 1.

are directed to both monomeric and dimeric forms of α3NC1.

Separation of α1NC1 and α2NC1 Dimers from α3NC1, α4NC1, and α5NC1 Dimers—NC1 dimers were fractionated by HPLC on a reversed-phase column, yielding fractions D₁ and D₂ (Fig. 2, panel A). These fractions were analyzed for their content of NC1 domains by ELISA. D₁ was found to be comprised of α1NC1 and α2NC1 dimers (panel B) and accounted for 70% of the dimer mass. Fraction D₂ was comprised of α3NC1 and α4NC1 dimers (panel C) and accounted for 30% of the dimer mass. D₂ also contained the dimers that bound GP antibodies.

Separation and Identification of D₁ and D₂ Components—The dimeric components comprising fractions D₁ and D₂ were resolved by two-dimensional electrophoresis. D₁ and D₂ each displayed a distinct pattern of more than 12 components (Fig. 3, panels A and F). Many of the D₁ components had mobilities identical to D₂ components, indicating that these were unresolved on two-dimensional electrophoresis of whole NC1 hexamer (Fig. 1).

The α chain identity of 23 D₁ and D₂ components was subsequently determined by NH₂-terminal sequence analysis, as summarized in Table I. D₁ components were identified as homodimers of either α1NC1 or α2NC1 because each showed a single NH₂-terminal sequence. α1NC1 and α2NC1 homodimers were previously established for EHS type IV collagen (12, 30). The multiplicity of components also indicated that both α1NC1 and α2NC1 homodimers each have several isoforms. Likewise, D₂ components were identified mainly as homodimers of α3NC1, α4NC1, and α5NC1 and their isoforms. In addition, two isoforms of an α3NC1-α1NC1 heterodimer, components 17 and 19, were detected, as well as two isoforms of α3NC1-α5NC1 heterodimer, components 18 and 23.

The α chain identity of 25 D₁ and D₂ components was also determined by immunoblotting analysis. The components were immunoblotted with synthetic peptide antibodies directed against α3NC1, α4NC1, and α5NC1 (Fig. 3, panels C-E and H-J, respectively) and polyclonal antibodies against α1NC1 and α2NC1 (data not shown). Their identities are summarized in Table I. The identities agreed with those derived by sequence analysis, except for components 13–15 in which α3NC1 was detected by blotting but in which its quantity was insufficient for detection by sequence analysis. Components 13–15 may contain small amounts of α3NC1-α4NC1 heterodimer or α3NC1 homodimer. The D₁ components did not bind anti-α3NC1, -α4NC1, or -α5NC1 antibodies, indicating that D₁ exclusively contained α1NC1 and α2NC1 homodimers. These results revealed that D₁ is comprised exclusively of homodimers of α1NC1 and α2NC1 and D₂ is comprised of mainly homodimers of α3NC1, α4NC1, and α5NC1. Relative abundance of dimers comprising the NC1 hexamer was estimated to be 70% α1 and α2NC1, 22% α3NC1 homodimer, 2% α4NC1 homodimer, 3% α5NC1 homodimer/heterodimer, and 3% α3-α1NC1 heterodimer, on the basis of relative amount of D₁ and D₂ determined by HPLC and on gel scans of D₂. The relative abundance of dimers comprising D₂ was determined to be 73% α3NC1, 8% α4NC1, 10% α5NC1, and 9% α1NC1.

The chain identity of dimeric components (GP antigen) binding GP antibodies was also determined by immunoblotting analysis. Binding was to certain D₂ components (Fig. 3, panels B and G). Their identities, as summarized in Table I, were found to be mainly homodimers of α3NC1 (components 16 and 20–22), an α3NC1-α1NC1 heterodimer, and possibly an α3NC1-α5NC1 heterodimer. In the latter case, binding to α5NC1 could not be excluded; however, this binding is unlikely because GP antibody does not bind to α5NC1 monomer as it does with α3NC1 monomer (compare panels B and C with panel F in Fig. 1). These results indicated that the dimeric form of the GP antigen is α3NC1 homodimers or α3NC1 containing heterodimers. A low titer of GP antibodies binding to D₁ was found by ELISA (Fig. 2), and the binding was to components 11 and 12 (Fig. 3) which correspond to two forms of α1NC1. This binding may reflect cross-reactivity between α3 and α1 NC1 dimers or to antibodies specific for α1NC1.

Specificity of GP Antibodies for GBM Constituents—The binding of GP antibodies to NC1 subunits, other domains of collagen IV, and other basement membrane components was measured by ELISA for patients LL and JB (Fig. 4). With respect to NC1 subunits, the binding was to α3NC1 monomer and dimer, and with respect to collagen IV domains, the binding was to the dissociated form of NC1 domain. Essentially no binding was found to other basement membrane constituents, i.e. fibronectin, laminin, heparan sulfate proteoglycan, entactin, or to collagen IV proteins from EHS tumor matrix. The absence of antibody binding to EHS collagen IV is consistent with our findings that α3NC1 is absent from the EHS preparation.α These results indicated that the target antigen of GBM for GP antibodies from these two patients is the NC1 domain of the α3 chain, and therefore, the antibodies were termed anti-α3(IV) antibodies.

α B. J. Wisdom, M. D. Hudson, S. Gunwar, M. E. Noelken, and B. G. Hudson, unpublished data.
In previous studies, the monomeric subunits of the NC1 hexamer of GBM collagen were identified as α1NC1, α2NC1, α3NC1, and α4NC1. In the present study, a fifth monomer, α5NC1, was identified, and the chain origin of 25 dimeric components was identified. These were established to be dimeric forms (disulfide-cross-linked) of each of the five NC1 domains. Dimers of α1NC1, α2NC1, α3NC1, and α4NC1 were established to be homodimers, each of which had several size and charge isoforms. The nature of the α5NC1 dimer and its isoforms was ambiguous, but it is likely to be a homodimer analogous to the other subunits. The findings confirmed the existence of the α5 chain in GBM as previously found by immunohistochemical studies (7). A small quantity of an α3NC1-α1NC1 heterodimer was also found, which substantiates earlier findings (5). These studies complete the identification of the chain origin of the multiple components comprising NC1 hexamer, and the findings provide direct evidence for the existence of all five α chains in GBM. The existence of yet another unidentified chain in GBM, however, cannot be excluded.

The five α chains indicate that in GBM the collagen IV suprastructure is comprised of several protomer subtypes. These differ with respect to the chain identity of the three chains forming the triple helix. The classical protomer is comprised of two α1 chains and one α2 chain (1, 2) and it is thought to be ubiquitous in mammalian basement membranes. Other protomer subtypes, whose chain organization is unknown, include those containing the α3, α4, α5 chains (4, 22). The subtypes may include homotrimers of each chain, as exemplified by α1 homotrimer found in cell culture (31) and in Drosophila (32), or heterotrimers of varying chain compositions.

In collagen IV suprastructure, protomers associate in a head-to-head (NC1-to-NC1) fashion to form dimers. This mode of association is well established for the classical protomer. However, this mode must hold true for the other protomers of α3, α4, and α5 chains, because the NC1 domain of each chain, when excised from the suprastructure by collagenase digestion, exists as an NC1 hexamer subunit. The existence of several protomer subtypes, however, implies that at least two possible kinds of protomer-protomer (NC1-to-NC1) associations are possible, i.e. protomers of like subtype associate forming homodimers or protomers of different subtypes associate forming heterodimers (22).

The present finding that the NC1 hexamer subunits are homodimers of the NC1 domains of α1,α2, α3, and α4 chains, and probably the α5 chain, implies a specificity of protomer-protomer association. The dimerization of subunits represents intermolecular disulfide cross-links that occur between adjoining protomers and which stabilize NC1-to-NC1 associations (2, 12, 30). That the subunits are homodimers suggests that virtually all of the protomer-protomer associations are between like subtypes. The existence of an α3NC1-α1NC1 heterodimer suggests that a small portion of α3-protomers are associated with the classical α1,α2-protomer. This specificity of association is supported by our previous finding of at least two distinct NC1 hexamers, one composed primarily of α1NC1 and α2NC1 subunits and the other primarily of α3NC1 subunits, as determined by immunoprecipitation studies (5). Potentially, the NC1 domain of each α chain possesses the structural information that specifies the chain composition of the protomer as well as the NC1-to-NC1 protomer-protomer association. In support of this contention, we found that the capacity for hexamer assembly is contained within the monomeric forms of the NC1 hexamer subunits (33).

The identification of dimeric components in the present study also firmly establishes size and charge isoforms as a structural feature for all five NC1 domains. Each of the homodimers has two size isoforms that differ in apparent molecular mass by about 5 kDa, and therefore we refer them as size isoforms and each size isoform has several charge isoforms. This feature is exemplified by α1NC1 dimers for which eight isoforms exist, i.e. two size isoforms with each having four charge isoforms (Fig. 3A). On the basis of these results, the two-dimensional patterns for NC1 hexamer of

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**FIG. 3. Two-dimensional electrophoresis analysis of dimeric subunits of fractions D1 and D2.** Gels in panels A and F were stained for protein with silver. Gels in panels B and G were immunoblotted with GP plasma-LL, panels C and H with α3NC1 antibody, D and I with α4NC1 antibody, and E and J with α5NC1 antibody. A one-dimensional SDS-PAGE gel is shown in the left section of each panel for reference. Two μg of sample was used for silver staining and 4–6 μg for immunoblots. The pI markers are indicated by arrowheads.

**DISCUSSION**

In previous studies, the monomeric subunits of the NC1 hexamer of GBM collagen were identified as α1NC1, α2NC1, α3NC1, and α4NC1. In the present study, a fifth monomer, α5NC1, was identified, and the chain origin of 25 dimeric components was identified. These were established to be dimeric forms (disulfide-cross-linked) of each of the five NC1 domains. Dimers of α1NC1, α2NC1, α3NC1, and α4NC1 were established to be homodimers, each of which had several size and charge isoforms. The nature of the α5NC1 dimer and its isoforms was ambiguous, but it is likely to be a homodimer analogous to the other subunits. The findings confirmed the existence of the α5 chain in GBM as previously found by immunohistochemical studies (7). A small quantity of an α3NC1-α1NC1 heterodimer was also found, which substantiates earlier findings (5). These studies complete the identification of the chain origin of the multiple components comprising NC1 hexamer, and the findings provide direct evidence for the existence of all five α chains in GBM. The existence of yet another unidentified chain in GBM, however, cannot be excluded.

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Charge isoforms were established previously for the monomeric forms of α1NC1, α2NC1, α3NC1, and α4NC1, and isoforms were postulated for the dimeric forms (22).
placenta and lung tissue can now be interpreted (22). The dimer subunits of these tissues also exhibit both size and charge isoforms. Thus, size and charge isoforms are a characteristic feature of the dimeric subunits for all tissues thus far examined. The molecular basis and biological importance of this feature remain unknown. The present study of NC1 dimers together with our previous ones of NC1 monomers (3, 5, 6, 15) establish the α3 chain as the target for circulating anti-GBM antibodies of patients with GP syndrome and the α3NC1 domain as the epitope site. This conclusion is further supported by a recent finding that the circulating antibodies from each of 37 patients with anti-GBM glomerulonephritis specifically bound to human α3NC1 monomer (34). The evidence in toto indicates that anti-collagen-α3(IV) antibodies are the anti-GBM autoantibodies causing glomerulonephritis and pulmonary hemorrhage.

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**Fig. 4.** Specificity of binding of GP antibodies from patients LL and JB to basement membrane components. The solid bars designate GP plasma-LL, striped bars designate GP plasma-JB, and stippled bars designate control serum. The various basement membrane components were: F, fibronectin; L, laminin; HSP, heparan sulfate proteoglycan; CIV, collagen IV from EHS tumor matrix. The collagenous domains were from bovine GBM collagen; the designations are: TH, triple helical domain (pepsin fragments); 7S, 7S domain; NC1, noncollagenous domain; and dNC1, denatured NC1. The NC1 subunits were from bovine GBM collagen IV. α1+α2NC1 dimers and α3NC1 dimer correspond to fractions D1 and D2 (Fig. 2), respectively. Fraction D2 is comprised of 73% α3NC1, 8% α4NC1, 9% α1NC1, and 10% α5NC1, and only α3 components react with GP antibody, as determined by Western blotting (Fig. 3).

**TABLE I**

| HPLC fraction*, component number* | NH2-terminal sequence | Chain identity by immunoblot* | GP antibody binding* |
|----------------------------------|-----------------------|-----------------------------|---------------------|
| D1                               |                       |                             |                     |
| 3, 4, 5, 6, 9, 10, 11, 12        | GPPGTPSVDHGFVTRHSQT   | α1NC1                       | −                   |
| 7, 2, 7, 8                       | GMPGRSISIGLYLVKHSQT   | α2NC1                       | −                   |
| D2                               |                       |                             |                     |
| 16, 20, 21, 22                   | GPPAAAGVMGFVTRHSQT    | α3NC1                       | ++++                |
| 18, 14, 15                       | GPPGFPGYLSFLVHLHVSQT  | α4NC1                       | ++                  |
| 17, 19                           | GPPAAAGVMGFVTRHSQT    | α5NC1                       | ++++                |
| 18, 23                           | GLXKGPGDTGPPAAGVMG    | α6NC1                       | ++++                |
| 24, 25                           | GPPGTSSVAHGLITRHSQT   | α7NC1                       | ++                  |

* NC1 dimer subunits were resolved into two fractions (D1 and D2) on a C18 column (Fig. 2). The relative amounts of D1 and D2 were 70 and 30%, respectively, based on the area of the peak in Fig. 2 as well as weight. 

* NC1 dimers were resolved by two-dimensional electrophoresis, and the components were designated by numbers 1–25 (Fig. 3). 

* Subunits were identified by comparing their sequences with those previously reported for α1NC1, α2NC1, α3NC1, and α4NC1 (6) and for α5NC1 (7, 8). 

* Immunoblots are shown in Fig. 3. Data for α1 and α2 are not shown. 

* Immunoblots with GP antibodies are shown in Fig. 3; + weakly intensive staining; ++++ strongly intensive staining; − absence of staining. 

* Sequences of components 18 and 23 correspond to that of α3aNC1 (6), whereas sequences of components 17, 19, 20, and 21 correspond to that of α3bNC1 (6). α3bNC1 is a truncated form of α3aNC1 in which the first 9 residues are deleted. α5NC1 sequences were compared with those previously reported (7, 8). The amount of α3bNC1 varied among different preparations of hexamer. The components 16, 20, 21, and 22 showed predominantly α3bNC1 sequences.
Basement Membrane Collagen

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