The Goodpasture Autoantigen

STRUCTURAL DELINEATION OF TWO IMMUNOLOGICALLY PRIVILEGED EPITOPES ON \( \alpha 3(IV) \) CHAIN OF TYPE IV COLLAGEN*

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The family of type IV collagen comprises six chains numbered \( \alpha 1 \) through \( \alpha 6 \). The \( \alpha 3(IV) \) NC1 domain is the primary target antigen for autoantibodies from patients with anti-basement membrane disease and Goodpasture syndrome. Earlier peptide studies suggested that the last 36 amino acids of the \( \alpha 3 \) NC1 domain probably contains one recognition site for Goodpasture autoantibodies, and an algorithm analysis of secondary structure from a later study predicted a second possible upstream epitope near the triple helix junction. We have used several analytic approaches to evaluate the likelihood of two immunologic epitopes for the Goodpasture antigen. In our first set of studies, peptide antibodies directed against these two putative regions co-inhibited Goodpasture autoantibodies binding to denatured human \( \alpha 3(IV) \) NC1 monomer by nearly 80%, with the helix-NC1 junction region of the \( \alpha 3 \) NC1 domain contributing 26% of the binding sites and the C-terminal region contributing the remaining 50%. Second, both of these candidate regions are normally sequestered within the associated \( \alpha 3(IV) \) NC1 hexamer but become more visible for binding by anti-peptide antibodies upon their dissociation, a property that is shared by the Goodpasture autoantibodies. Third, segment deletions of recombinant \( \alpha 3 \) NC1 domain further confirmed the presence of two serologic binding sites. Finally, we looked more closely at the C-terminal binding region of the \( \alpha 3(IV) \) NC1 domain. Since the lysines in that region have been previously advanced as possible contact sites, we created several substitutions within the C-terminal epitope of the \( \alpha 3 \) NC1 domain. Substitution of lysines to alanines revealed lysines 219 and 229 as essential for antibody binding to this distal site; no lysines were present in the NC1 part of the helix-NC1 junction region. Substitutions involving arginine and cysteines to alanines in the same C-terminal region did not produce significant reductions in antibody binding. In summary, our findings characterize two Goodpasture epitopes confined to each end of the \( \alpha 3 \) NC1 domain; one is lysine-dependent, and the other is not. We propose, as a hypothetical model, that these two immunologically privileged regions fold to form an optimal pathogenic structure within the NC1 domain of the \( \alpha 3 \) chain. These sites are subsequently concealed by NC1 hexamer assembly of type IV collagen.

Goodpasture syndrome is an autoimmune disease characterized typically by rapidly progressive glomerulonephritis and pulmonary hemorrhage mediated by anti-GBM autoantibodies (1-11). The principal target for these antibodies is the NC1 domain of the \( \alpha 3(IV) \) chain of type IV collagen (1, 3, 12-15). Type IV collagen constitutes the major protein of mammalian basement membranes (1, 16-18). It plays a role in providing tensile strength and scaffolding for the binding and alignment of other basement membrane molecules, like laminin, entactin, proteoglycan, and fibronectin (19-22).

Type IV collagen is composed of six genetically distinct \( \alpha \)-chains (\( \alpha 1-\alpha 6 \)) (1, 12, 15, 23-31). The type IV collagen promoter is characterized by three distinct structural domains: the amino-terminal collagenase-resistant collagenous 7S domain, the carboxyl-terminal noncollagenous NC1 domain, and the major triple helical region between the terminal domains (1). Bacterial collagenase solubilizes assembled type IV protomers, digesting the triple helix and leaving NC1 hexamers and 75 resistant fragments (3). Detailed studies of the NC1 domain of type IV collagen from several tissues indicate that these hexamers are comprised of dimers (45,000-55,000) and monomers (24,000-28,000), the ratio of which varies from tissue to tissue (3, 13, 32).

The \( \alpha 3(IV) \) chain has also been cloned from several different species and localized to human chromosome 2 (24, 33). The full-length amino acid sequence of the \( \alpha 3(IV) \) NC1 domain reveals several structural similarities to the other chains of type IV collagen as well as some distinct differences (34). Some regions of the NC1 domain, for example, have been implicated in the activation of polymorphonuclear neutrophils (35). Other mutations in the \( \alpha 3(IV) \) chain have also been detected as polymorphisms in patients with autosomal recessive Alport syndrome (1, 36), and in Alport patients with post-transplant anti-GBM nephritis, the target for their anti-GBM antibodies is also the \( \alpha 3(IV) \) NC1 domain (1, 37, 38). Recently, the human \( \alpha 3(IV) \) NC1 domain has been expressed in recombinant form and shown to selectively bind Goodpasture autoantibodies (39). Using this recombinant protein and site-specific antibodies, we have further characterized the Goodpasture antibody epitopes.

MATERIALS AND METHODS

Preparation of Recombinant Human \( \alpha 3(IV) \) NC1 Domain and Generation of Mutant Sequences—Various recombinant modifications, in-

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‡ The abbreviations used are: NC1, noncollagenous domain 1; GP, Goodpasture; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; GBM, glomerular basement membranes.
 immunizations, followed by three subcutaneous injections of 500 μg of the protein.

The anti-α3(IV) NC1 Alport allantibodies were previously described (37). Immuno blotting—SDS-PAGE in one dimension was carried out with 14% gels and the discontinuous buffer system of Laemmli (45). Western blotting was performed according to Burnette (46) with minor modifications. Briefly, the separated proteins were transferred to nitrocellulose paper and blocked with 2% bovine serum albumin for 30 min on a shaker at room temperature. After blocking the remaining binding sites, the blot was washed thoroughly with washing buffer (0.1% Tween 20) and incubated with a primary antibody at an appropriate dilution in incubation buffer (phosphate-buffered saline, 1% bovine serum albumin). The incubation was carried out at room temperature overnight on a shaker. Subsequently, the blot was washed thoroughly with washing buffer and incubated with secondary antibody conjugated to horseradish peroxidase for 1 h at room temperature on a shaker. The secondary antibody conjugated to alkaline phosphatase at 1:5000 dilution with alkaline phosphatase substrate buffer containing disodium nitrophenyl phosphate (5 mg/ml) was added. After color development, the absorbance was measured using micro-

### TABLE I

| α3 mutants | Primers |
|------------|---------|
| α3n-26     | 5'-Primer: 5'-CGGGATCCCTCAACACCAGCAATT-3' |
| α3n-26/α3-36 | 3'-Primer: 5'-CGAAAGTCTTGCAGTCTGTTTTCCTCAT-3' |
|            | 3'-Primer: 5'-CGGGATCCCTCAACACCAGCAATT-3' |
| α3n-26/α3KK | 5'-Primer: 5'-CGGGATCCCTCAACACCAGCAATT-3' |
| α3/C.K.206 | Outer 5'-primer: 5'-CGGGATCCCTTGTTTTAGAAAGAAAATCACTGAG-3' |
|            | Outer 3'-primer: 5'-CTGACACGCCGATATGGTTTTAGAAAGAAAATCACTGAG-3' |
| α3/C.K.213 | Internal 5'-primer: 5'-CGGGATCCCTTGTTTTAGAAAGAAAATCACTGAG-3' |
|            | Internal 3'-primer: 5'-CTGACACGCCGATATGGTTTTAGAAAGAAAATCACTGAG-3' |
| α3/C.K.219 | Outer 5'-primer: 5'-CGGGATCCCTTGTTTTAGAAAGAAAATCACTGAG-3' |
|            | Outer 3'-primer: 5'-CTGACACGCCGATATGGTTTTAGAAAGAAAATCACTGAG-3' |
| α3/C.K.229 | 5'-Primer: 5'-CGGGATCCCTTGTTTTAGAAAGAAAATCACTGAG-3' |
| α3/C.K.230 | 3'-Primer: 5'-CGGGATCCCTTGTTTTAGAAAGAAAATCACTGAG-3' |
| α3/KK      | 3'-Primer: 5'-CGGGATCCCTTGTTTTAGAAAGAAAATCACTGAG-3' |
|            | 3'-Primer: 5'-CGGGATCCCTTGTTTTAGAAAGAAAATCACTGAG-3' |
| α3/KK/α3KK | 5'-Primer: 5'-CGGGATCCCTTGTTTTAGAAAGAAAATCACTGAG-3' |
| α3/R.223   | Outer 5'-primer: 5'-CGGGATCCCTTGTTTTAGAAAGAAAATCACTGAG-3' |
|            | Outer 3'-primer: 5'-CGGGATCCCTTGTTTTAGAAAGAAAATCACTGAG-3' |
|            | Internal 5'-primer: 5'-TTAGAAAAAATAATAAGTGCCTGTCAGGTGTGCATGAAG-3' |
|            | Internal 3'-primer: 5'-CCTATTCCATAAACTGTGGCCGCTGGGGAATTAGAAAAA-3' |
|            | Internal 3'-primer: 5'-TTAGAAAAAATAATAAGTGCCTGTCAGGTGTGCATGAAG-3' |
|            | Internal 3'-primer: 5'-CCTATTCCATAAACTGTGGCCGCTGGGGAATTAGAAAAA-3' |

*Used the template resulting from α3/C.K.219 polymerase chain reaction.

The Goodpasture epitope—Human and bovine NC1α3(IV) NC1 hexamers (41) and bovine α3(IV) NC1 hexamers were prepared from kidneys by a standard procedure (13, 42, 43). Some of the associated NC1 hexamers were solubilized in 6 M guanidine-HCl, 50 mM Tris-Cl, pH 7.5, and incubated for 10 min at 85°C. This procedure separates the associated NC1 hexamers into a denatured form consisting of dimers and monomers.

Preparation of Peptide Antibodies—The polyclonal anti-α3n-α3-36 antibody, anti-α3n-18 antibody, and anti-NC1 antibodies were made in rabbits. The 36-amino acid peptide in the C-terminal region and the 18-amino acid peptide in the N-terminal region were synthetically prepared by the t-boc method (41). The 18 amino acids in the triple helix-NC1 junction were chosen because Quinones et al. hypothesized the involvement of these amino acids in the Goodpasture epitope (44). These 18 amino acids are contained within the 26 amino acids we chose to delete in the present study. The peptides were conjugated to keyhole limpet hemocyanin, and rabbits were given three subcutaneous injections of a 250-μg equivalent of each peptide (27). The antiserum was collected after 6 weeks for specific antibodies using bovine type IV collagen NC1 domains and recombinant type IV collagen NC1 domains (data not shown) (39). Anti-NC1 hexamer antibody was also prepared in rabbits using three subcutaneous injections of 500 μg of the protein.

In conclusion, the design and preparation of the second antibody conjugated to alkaline phosphatase prepared in a 1,500 dilution with phosphate-buffered saline containing 1% bovine serum albumin. The plates were incubated for 2 h at 37°C or overnight at room temperature. Upon coating, the plates were washed three times at intervals of 5 min with 0.15 M NaCl and 0.05% Tween 20 (washing buffer). After washing, the plates were blocked with 2% bovine serum albumin for 30 min at 37°C. After blocking, the plates were again washed and then incubated with primary antibody in an appropriate dilution with phosphate-buffered saline containing 1% bovine serum albumin. The plates were incubated for 1 h at 37°C. After primary antibody incubation, the plates were again washed and then incubated with the secondary antibody conjugated to alkaline phosphatase at 3,000 dilution with phosphate-buffered saline. The plates were incubated for 1 h at 37°C. After washing, the plates were blocked with 2% bovine serum albumin for 30 min at 37°C. After blocking, the plates were again washed, and then incubated with disodium p-nitrophenyl phosphate (5 mg/ml) was added. After color development, the absorbance was measured using micro-

**TABLE I**

List of polymerase chain reaction mutagenesis primers

| α3 mutants | Primers |
|------------|---------|
| α3n-26     | 5'-Primer: 5'-CGGGATCCCTCAACACCAGCAATT-3' |
| α3n-26/α3-36 | 3'-Primer: 5'-CGAAAGTCTTGCAGTCTGTTTTCCTCAT-3' |
|            | 3'-Primer: 5'-CGGGATCCCTCAACACCAGCAATT-3' |
| α3n-26/α3KK | 5'-Primer: 5'-CGGGATCCCTCAACACCAGCAATT-3' |
| α3/C.K.206 | Outer 5'-primer: 5'-CGGGATCCCTTGTTTTAGAAAGAAAATCACTGAG-3' |
|            | Outer 3'-primer: 5'-CTGACACGCCGATATGGTTTTAGAAAGAAAATCACTGAG-3' |
| α3/C.K.213 | Internal 5'-primer: 5'-CGGGATCCCTTGTTTTAGAAAGAAAATCACTGAG-3' |
|            | Internal 3'-primer: 5'-CTGACACGCCGATATGGTTTTAGAAAGAAAATCACTGAG-3' |
| α3/C.K.219 | Outer 5'-primer: 5'-CGGGATCCCTTGTTTTAGAAAGAAAATCACTGAG-3' |
|            | Outer 3'-primer: 5'-CTGACACGCCGATATGGTTTTAGAAAGAAAATCACTGAG-3' |
| α3/C.K.229 | 5'-Primer: 5'-CGGGATCCCTTGTTTTAGAAAGAAAATCACTGAG-3' |
| α3/C.K.230 | 3'-Primer: 5'-CGGGATCCCTTGTTTTAGAAAGAAAATCACTGAG-3' |
| α3/KK      | 3'-Primer: 5'-CGGGATCCCTTGTTTTAGAAAGAAAATCACTGAG-3' |
|            | 3'-Primer: 5'-CGGGATCCCTTGTTTTAGAAAGAAAATCACTGAG-3' |
| α3/KK/α3KK | 5'-Primer: 5'-CGGGATCCCTTGTTTTAGAAAGAAAATCACTGAG-3' |
| α3/R.223   | Outer 5'-primer: 5'-CGGGATCCCTTGTTTTAGAAAGAAAATCACTGAG-3' |
|            | Outer 3'-primer: 5'-CGGGATCCCTTGTTTTAGAAAGAAAATCACTGAG-3' |
|            | Internal 5'-primer: 5'-TTAGAAAAAATAATAAGTGCCTGTCAGGTGTGCATGAAG-3' |
|            | Internal 3'-primer: 5'-CCTATTCCATAAACTGTGGCCGCTGGGGAATTAGAAAAA-3' |
|            | Internal 3'-primer: 5'-TTAGAAAAAATAATAAGTGCCTGTCAGGTGTGCATGAAG-3' |
|            | Internal 3'-primer: 5'-CCTATTCCATAAACTGTGGCCGCTGGGGAATTAGAAAAA-3' |

*Used the template resulting from α3/C.K.219 polymerase chain reaction.
The a3(IV) NC1 domain and its mutants expressed in Escherichia coli as fusion proteins encoded by pDS-MCS. The a3(IV) NC1 domain and all the deletions and mutant proteins are summarized in the list below the plasmid picture. All the recombinant proteins were expressed with a six-histidine tag (underlined). The histidine leader in each construct was extended by one lysine residue created during the reformation of the S-BamHI site. The a3 chain of type IV collagen is a polypeptide of 1670 amino acids (34). The NC1 domain starts at residue 1439 and ends at amino acid 1670 (232 amino acids). The present study was conducted on an E. coli expressed recombinant fragment of a3(IV) chain starting at amino acid 1427 and ending at residue 1670. This fragment contains 12 amino acids of the triple helix-NC1 junction Gly-X-Y sequence and the entire NC1 domain of the a3(IV) chain. In the present study, the residue 1439 of the a3(IV) chain was designated as amino acid 1, the first amino acid of the a3(IV) NC1 domain.

ELISA autoreader at 405 nm.

Inhibition ELISA was performed as before (41, 43) with slight modification. The ELISA plates (NUNC-immuno-plates-Maxisorp, Nunc, Denmark) were coated with 25 ng of denatured human a3(IV) NC1 monomer. The plates were coated overnight at room temperature. Upon washing and blocking the plates with bovine serum albumin as described earlier, the plates were incubated with Goodpasture antibodies (1:1000) containing increasing amounts of either recombinant a3(IV) NC1 or the mutants. The increasing concentrations were 1:2, 1:4, 1:6, and 1:10 of the concentration for 25 ng of human Goodpasture antibodies (saturating antibody concentration). The plates were then washed before development. The control antibodies did not bind to either form (Fig. 2). This effect was also observed with the Goodpasture antibodies. Control antibodies did not bind to either form of the antigen. Antibodies generated against the 160-kDa associated bovine NC1 hexamer revealed similar binding to both forms of the bovine NC1 hexamer (Fig. 2). As an additional control, an interesting effect was observed with anti-a3(IV) NC1 antibody obtained from an Alport patient following renal transplantation. These antibodies are made by some Alport syndrome patients with Alport syndrome develop anti-a3(IV) NC1 antibodies upon renal allograft transplantation (23) showed the opposite effect. The anti-associated NC1 hexamer antibody shows no change in binding under either condition. The control antibodies did not reveal any significant binding to either form of the antigen.

RESULTS

Previous reports have suggested two regions of the a3(IV) NC1 domain as possible candidates for the Goodpasture epitope (41, 44). An interaction site for antibody was initially proposed for the last 36 amino acids of the a3 NC1 domain (41) based on peptide analysis. A year later a structural algorithm in another study predicted a second interaction site near the triple helix-NC1 junction of the a3 NC1 domain (2).

Cryptic Properties of the Goodpasture Epitope—The NC1 hexamer of type IV collagen exists in its native, associated form as a 160-kDa molecule (32, 43). This hexamer is comprised of monomeric and dimeric subunits held together by noncovalent bonds (1–3, 32, 43). Previous studies have shown that Goodpasture autoantibodies bind to the associated NC1 hexamer weakly (43), but upon dissociation of the hexamer by denaturants or low pH (denatured NC1 hexamer), the binding of antibody increases by severalfold.

We prepared antibodies to two regions of the a3 NC1 domain (a3/c-36 and a3/n-18 regions) that may form the putative Goodpasture epitopes. Both of these antibodies bind 3–4-fold better to the denatured bovine NC1 hexamer than to the associated form (Fig. 2). This effect was also observed with the Goodpasture antibodies. Control antibodies did not bind to either form of the antigen. Antibodies generated against the 160-kDa associated bovine NC1 hexamer revealed similar binding to both forms of the bovine NC1 hexamer (Fig. 2). As an additional control, an interesting effect was observed with anti-a3(IV) NC1 antibody obtained from an Alport patient following renal transplantation. These antibodies are made by some Alport patients in response to exposure to normal a3(IV) chains in the renal allograft; patients develop severe anti-GBM nephritis and reject their transplanted kidneys. The major target for these anti-GBM autoantibodies is also the a3(IV) NC1 domain (37, 38, 43). We found that these autoantibodies, in contrast to the Goodpasture antibody, bind strongly to the associated form of bovine NC1 hexamer and 4–5-fold less against denatured bovine NC1 hexamer.
cumulative effect of these two peptide antibodies, a double antibody inhibition was performed (Fig. 3, panel C). In this experiment, the best binding dilution of the anti-α3-C-36 peptide antibody was used as a constant, and different dilutions of anti-α3-n-18 peptide antibody as a variant. The results show an increasing inhibitory effect of the anti-α3-n-18 peptide antibody leveling off at about 76% inhibition of the Goodpasture antibody (GP-1) binding. Curiously, the inhibitory effect of the anti-α3-n-18 peptide antibody was maximal at 1:200 dilution, whereas the anti-α3-n-18 peptide antibody binding to the denatured human α3(IV) NC1 monomer was not maximal at that dilution (Fig. 3, panel A). The probable explanation for this difference is some additional spatial restriction imposed on the α3(IV) NC1 due to the binding of the anti-α3-c-36 blocking antibody.

Clarification of Autoantibody Binding Regions within the α3(IV) NC1 Domain—To analyze the role of α3 NC1/n-26 and α3 NC1/c-36 regions in antibody binding, recombinant α3(IV) NC1 domains were generated that lacked the region α3 NC1/n-26 (α3/n-26) or both α3 NC1/n-26 and α3 NC1/c-36 regions (α3/n-26/c-36). These deletion and point mutants were analyzed by SDS-PAGE and found to be of the predicted size (Fig. 4, panel A). Immunoblotting and ELISA analysis of these mutants using three different Goodpasture antibodies (GP-1, GP-2, and GP-3) show a substantial reduction in binding for α3/n-26 and a further reduction for the mutant α3/n-26/c-36 (Fig. 4, panel C).

Chemical modification studies previously suggested that lysines may be necessary for Goodpasture autoantibody binding in the C-terminal binding region (41). Therefore, lysine 229 and lysine 230 were mutated to alanines along with the deletion of α3 NC1/n-26 region. This mutant (α3/n-26/cKK) revealed significant reduction in its binding to Goodpasture antibodies, similar to mutant α3/n-26/c-36 (Fig. 4, panel C). We further analyzed the role of individual lysines within the region. There are a total of six lysines present in the α3(IV) NC1 domain, and five of them are present in the α3 NC1/c36 region. The α3 NC1/n26 does not contain any lysines in the NC1 part of this region. Although there are two lysines present in the triple helical part of this region, previous studies suggest that these may not be involved in the Goodpasture autoantibody binding (27). Collagenase digestion of GBM cleaves the α3(IV) NC1 region at two places, one at the triple helix-NC1 border region and the other at site nine amino acids (including two lysines) away from the NC1 region, into the triple helix (27). These two forms of α3(IV) NC1 domains bind Goodpasture autoantibodies strongly (27). Therefore, the two lysines in the triple helical region of the α3 NC1/n26 do not seem important in the Goodpasture autoantibody binding. Hence, in the present study these lysines were not mutated.

The lysines in the α3 NC1/c36 region (Lys-206, -213, -219, -229, and -230) were individually substituted in the α3 NC1/c36 region to alanines. SDS-PAGE analysis was performed to check for the predicted size (Fig. 4, panel B). Immunoblotting and ELISA experiments were performed on these mutants using the same Goodpasture antibodies as in the experiment shown in Fig. 4, panel C. Replacement of lysine 219 (α3/c-K.219) or lysine 229 (α3/c-K.229) resulted in a steep reduction in binding with Goodpasture antibodies (Fig. 4, panel D). Replacement of lysines 206, 213, and 230 did not produce any significant change in their binding to Goodpasture antibodies when compared with recombinant α3(IV) NC1 domain. Additionally, an arginine substitution (arginine 223) to alanine did not change in Goodpasture antibodies binding either.

In order to determine if lysines 219 and 229 have a synergistic effect on binding to antibody, two new mutants were

Fig. 3. Inhibition analysis with site-specific anti-α3(IV) NC1 antibodies. Panel A, the plates were coated with 25 ng of denatured human α3(IV) NC1 hexamer and a dilution curve was obtained for the anti-α3-C.36 peptide antibody (solid circles). Panel B, each of the two peptide antibodies were assayed for their capacity to inhibit Goodpasture autoantibody binding (GP-1) to the antigen. The dilutions refer to the different peptide antibody dilutions used in the assay. The rectangles represent the anti-α3-C.36 peptide antibody, and the circle represents anti-α3-N.18 peptide antibody. The Goodpasture antibody binding to the human α3(IV) NC1 was calculated as 100% for these experiments. Panel C, a double antibody inhibition was performed to address the cumulative effect of the two α3(IV) NC1-specific peptide antibodies in inhibiting the Goodpasture antibodies binding to the antigen. The maximal binding dilution of 1:50 for Goodpasture antibody was used in this experiment (data not shown).

Inhibition Studies with Site-specific Anti-α3(IV) NC1 Peptide Antibodies—Dilution curves for anti-α3-c-36 peptide antibody and anti-α3-n-18 peptide antibody against human α3(IV) NC1 monomer were performed in Fig. 3, panel A. These antibodies were then used in an inhibition ELISA to demonstrate their effect in blocking the binding of Goodpasture antibody (GP-1) to human α3(IV) NC1 monomer. The anti-α3-c-36 peptide antibody exhibits a maximum of 48% inhibition, and the anti-α3-n-18 peptide antibody shows 41% inhibition (Fig. 3, panel B). These results further support the notion that the C-terminal and the N-terminal end regions of the α3(IV) NC1 domain are critical to Goodpasture autoantibody binding. To evaluate the
designed. One mutant involved the substitution of three lysines (positions 219, 229, and 230) to alanines (α3/c-KKK), and the other mutant involved lysines 229 and 230 (α3/c-KK) also changed to alanines. Both of these mutants showed similar reduction in binding to antibody as the substitutions in α3/c-K.219 and α3/c-K.229. Therefore, the effects of lysines 219 and 229 seem independent and are not additive, since mutation of both at the same time (α3/c-KKK) did not show any noticeable difference in binding as compared with mutants α3/c-K.219 and α3/c-K.229 alone.

Analysis of Recombinant α3(IV) NC1 Mutants Using Denatured Human α3(IV) NC1 Monomer in Inhibition ELISA—The recombinant α3(IV) NC1 mutants that exhibited reduced binding with Goodpasture antibodies (Fig. 4, panels C and D) were further analyzed for their capacity to inhibit antibody binding to denatured human α3(IV) NC1 monomer. Since all the Goodpasture antibodies used show a similar effect, only GP-1 was for this experiment. Inhibition curves for the recombinant α3(IV) NC1 and its mutants are presented in Fig. 5. Recombinant α3(IV) NC1 inhibits Goodpasture antibody binding to denatured human α3(IV) NC1 monomer by 55% at saturating concentration. Inhibition curves for a representative group of mutants are also depicted; mutants α3/n-26/c-36 and α3/c-K.229 show only 18 and 29% inhibition, respectively, as compared with normal recombinant antigen. The control recombinant α6(IV) NC1 domain revealed <10% inhibition. In Fig. 6, the competitive inhibition of antibody binding to denatured...
human α3(IV) NC1 monomer observed with the recombinant α3(IV) NC1 was assigned a maximum value of 100%. All the inhibition values observed for the mutants were expressed relative to the recombinant; the largest inhibition was seen for mutant α3n-26/c-36 at 32.7%. r-α6, recombinant α6; r-α3, recombinant α3.

### DISCUSSION

Immunologic epitopes like those comprising the Goodpasture antigen have been studied in a variety of systems. Epitopes for which the complete structures have been elucidated by x-ray crystallography typically contain between 15 and 22 residues contributed from different regions of a target protein (49). This is consistent with the view that while the primary structures of native epitopes are often discontinuous (49–52), two- or three-dimensional folding creates a conformational or multiligated determinant. A recent study using a similar approach to ours identified two nonlinear domains of apoB100 as key parts of a conformational or multiheaded native epitopes are often discontinuous (49–52), two- or three-dimensional folding creates a conformational or multiheaded determinant. A recent study using a similar approach to ours identified two nonlinear domains of apoB100 as key parts of a conformational or multiheaded determinant.

In the present study, we have further distinguished two important interaction sites for Goodpasture autoantibody within the α3(IV) NC1 domain using serologic analyses and recombinant mutagenesis. One site resides among the last 36 amino acids, with lysines 219 and 229 being particularly necessary, and a second site lies within the first 26 amino acids of the triple helix-NC1 junction. These results bring together and solidify two previous suggestions regarding the determinants of the Goodpasture epitope (41, 44) and advance the notion that Goodpasture antibody may recognize an internalized structure.

The α3(IV) NC1 hexamers found in fragments of digested basement type IV collagen bind Goodpasture autoantibodies to only a limited extent, and these hexamers do not induce Goodpasture-like syndrome in rabbits (54); further denaturation of the α3(IV) NC1 hexamers into monomer and dimer subunits by denaturants, however, greatly increases the binding of antibody, and these isolated α3(IV) NC1 dimers induce Goodpasture disease in rabbits. The poor recognition of hexamer prior to denaturation suggests that candidate Goodpasture epitopes are immunologically privileged within the normal hexamer configuration. This privilege is not extended to binding sites recognized by anti-α3(IV) alloantibodies in AIport patients with anti-basement membrane disease following renal transplantation.

Our experiments with anti-peptide antibodies to the Goodpasture antigen, directed toward the α3 NC1/n26 and the α3 NC1/n36 regions, further support the notion of internalized or cryptic epitopes comprising the Goodpasture autoantigen. Anti-peptide antibodies to both regions bind weakly to the associated α3(IV) NC1 hexamer but strongly against hexamer denatured into monomer and dimers. Furthermore, these antibodies cumulatively inhibit Goodpasture autoantibodies binding to monomers by 75%. Taken together, these experiments suggest that the epitopes within these two ends of the α3(IV) NC1 domain are probably the most important regions.

Exposure of such privileged epitopes by smoke, oxidants, organic solvents, or infection, as a hypothesis, may modify collagen interactions in basement membranes in situ that facilitate subsequent inflammation and further opportunity for more antibody accumulation in organ tissues (48).

Inferring from the results obtained here, we propose as a hypothetical model that two immunologically privileged regions fold to form an optimal antigenic structure within the NC1 domain of the α3 chain. These Goodpasture sites are subsequently concealed by the normal assembly of type IV collagen into hexamers.

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