Hydrophobic Amino Acid Residues Are Critical for the Immunodominant Epitope of the Goodpasture Autoantigen

A MOLECULAR BASIS FOR THE CRYPTIC NATURE OF THE EPITOPE*

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Goodpasture (GP) autoimmune disease is caused by autoantibodies to type IV collagen that bind to the glomerular basement membrane, causing rapidly progressing glomerulonephritis. The immunodominant GP$_A$ autoepitope is encompassed by residues 17–31 (the EA region) within the noncollagenous (NC1) domain of the a3(IV) chain. The GP epitope is cryptic in the NC1 hexamer complex that occurs in the type IV collagen network found in tissues and inaccessible to autoantibodies unless the hexamer dissociates. In contrast, the epitope for the Mab3 monoclonal antibody is also located within the EA region, but is fully accessible in the hexamer complex. In this study, the identity of residues that compose the GP$_A$ autoepitope was determined, and the molecular basis of its cryptic nature was explored. This was achieved using site-directed mutagenesis to exchange the a3(IV) residues in the EA region with the corresponding residues of the homologous but non-immunoreactive a1(IV) NC1 domain and then comparing the reactivity of the mutated chimeras with GP$_A$ and Mab3 antibodies. It was shown that three hydrophobic residues (Ala$_{18}$, Ile$_{19}$, and Val$_{27}$) and Pro$_{28}$ are critical for the GP$_A$ autoepitope, whereas two hydrophilic residues (Ser$_{21}$ and Ser$_{31}$) along with Pro$_{28}$ are critical for the Mab3 epitope. These results suggest that the cryptic nature of the GP$_A$ autoepitope is the result of quaternary interactions of the a3, a4, and a5 NC1 domains of the hexamer complex that bury the one or more hydrophobic residues. These findings provide critical information for understanding the etiology and pathogenesis of the disease as well as for designing drugs that would mimic the epitope and thus block the binding of GP autoantibodies to autoantigen.

Goodpasture (GP)$^1$ disease is defined by rapidly progressive glomerulonephritis, with or without lung hemorrhage, which is caused by autoantibodies targeted to type IV collagen of the glomerular and alveolar basement membranes. Left untreated, the disease is potentially lethal. If diagnosed early, GP patients can be treated by immunosuppression and plasma exchange to remove the toxic autoantibodies. This therapy has side effects, including a weakening of the natural defenses; thus, a more specific therapy is highly desirable. A detailed knowledge of the epitope would facilitate the development of therapies that selectively remove, neutralize, or prevent synthesis of the pathogenic autoantibodies and provide a foundation for studies to determine the etiology of the disease.

The GP autoantigen is the a3 chain of type IV collagen (1, 2), one of the six chains (a1–a6) that compose type IV collagen (3). The GP autoepitopes are conformational and reside within the 232-residue long noncollagenous (NC1) domain at the C-terminus of the a3(IV) chain. Three chains of type IV collagen assemble into triple-helical protomers (molecule) that further interact with each other at the amino and carboxyl termini to form supramolecular networks. In the glomerular basement membrane, which is the main target of autoantibodies, the a3(IV) chain associates with the a4(IV) and a5(IV) chains to form a cross-linked a3/a4/a5(IV) network (4). The GP epitopes are cryptic in the a3/a4/a5 NC1 hexamer complex formed by the interaction of two triple-helical protomers through the NC1 domains (Fig. 1, top). As a result, the epitopes are inaccessible for binding of autoantibodies unless the hexamer dissociates (5–7). Unmasking of previously hidden GP epitopes is thought to be of fundamental importance for understanding the etiology and pathogenesis of GP disease.

Two conformational GP autoepitopes have recently been mapped to two regions of the a3(IV) NC1 domain, designated E$_A$ and E$_B$, by homolog-scanning mutagenesis using chimeric a1/a3 NC1 domains in which the non-immunoreactive a1 NC1 domain was used as a scaffold for exchanging short homologous a1 sequences with a3 NC1 segments to ensure correct folding of the epitope. The immunodominant autoepitope, designated GP$_A$ (7), has been localized to the N-terminal third of the a3(IV) NC1 domain (8, 9) and specifically to residues 17–31, designated the E$_A$ region (7, 10, 11). Autoantibodies specific for the E$_A$ region, designated GP$_A$*, are believed to play an important role in the pathogenesis of GP disease because they are the predominant subpopulation (60–65%) in all sera and have high affinity for autoantigen (7). Moreover, high titers of GP$_A$ antibodies are correlated with an unfavorable disease outcome (9). A second autoepitope, designated GP$_B$ (7), was also identified in the central portion of the a3(IV) NC1 domain (9) and was further mapped to residues 127–141, designated the E$_B$ region (7, 10).

In contrast to the cryptic GP autoepitopes, the epitope for Mab3 monoclonal antibody, which is also localized to the E$_A$ and E$_B$ regions, is fully accessible even in the NC1 hexamer (7).
Molecular Basis for Cryptic Nature of Goodpasture Autoepitope

These observations led to the hypothesis that only certain amino acid residues of the $E_A$ and $E_B$ regions constitute the $GP_A$ and $GP_B$ autoepitopes and that their cryptic nature in the NC1 hexamer complex is a result of either direct interactions with or close proximity to other NC1 domains in the hexamer, which prevents the access of autoantibodies. Moreover, these critical residues must be distinctly different from those that constitute the Mab3 epitope, which are accessible on the surface of the hexamer complex.

The aim of this study was to identify which residues compose the $GP_A$ autoepitope and to explore the molecular basis of its cryptic nature. This was accomplished using homolog-scanning mutagenesis to change the $a3(IV)$-specific residues within the $a3(IV)$ NC1 domain. These regions jointly form the epitope for Mab3. The GP epitopes (diagonal lines) are cryptic in the NC1 hexamer complex and inaccessible for binding of autoantibody (shown for GP antibodies; right), but they are exposed upon dissociation of the hexamer into subunits, allowing binding of the autoantibody. In contrast, the Mab3 epitope (solid black) is accessible in both the hexamer and dissociated (monomer) form of the $a3(IV)$ NC1 domain (left). Hence, in the NC1 hexamer complex, the $E_A$ and $E_B$ regions contain certain inaccessible residues that are critical for the GP epitopes and other accessible residues that are critical for the Mab3 epitope (7).

**Fig. 1.** Model representing the location within the native NC1 hexamer complex of the cryptic GP autoepitopes and of the exposed Mab3 epitope of the $a3(IV)$ NC1 domain. In the type IV collagen networks found in vivo, two triple-helical collagen protomers interact through their carboxy-terminal ends, forming an NC1 hexamer complex (top). Two conformational GP epitopes, designated $GP_A$ and $GP_B$, have been localized to the $E_A$ and $E_B$ regions of the $a3(IV)$ NC1 domain. These regions jointly form the epitope for Mab3. The GP epitopes (diagonal lines) are cryptic in the NC1 hexamer complex and inaccessible for binding of autoantibody (shown for GP antibodies; right), but they are exposed upon dissociation of the hexamer into subunits, allowing binding of the autoantibody. In contrast, the Mab3 epitope (solid black) is accessible in both the hexamer and dissociated (monomer) form of the $a3(IV)$ NC1 domain (left). Hence, in the NC1 hexamer complex, the $E_A$ and $E_B$ regions contain certain inaccessible residues that are critical for the GP epitopes and other accessible residues that are critical for the Mab3 epitope (7).

**Table I**

| Mutant | Mutation Primer (5’ → 3’) |
|--------|---------------------------|
| M1     | Thr$^{17}$ → Ile           |
| M2     | Ala$^{18}$ → Asp           |
| M3     | Ile$^{21}$ → Asp           |
| M4     | Ser$^{24}$ → Gln           |
| M5     | Glu$^{26}$ → Ser           |
| M6     | Val$^{27}$ → Lys           |
| M7     | Pro$^{28}$ → Ile           |
| M8     | Ser$^{31}$ → His           |

Primer (5’ → 3’)

|   |   |   |   |   |
|---|---|---|---|---|
|   |   |   |   |   |

The cryptic nature of the $GP_A$ autoepitope is the result of quaternary interactions among the $a3(IV)$, $a4(IV)$, and $a5(IV)$ NC1 domains of the $a3(a4a5)$ hexamer complex that bury the one or more hydrophobic residues. These findings provide critical information for understanding the etiology and pathogenesis of the disease as well as for designing drugs that would mimic the epitope and thus block the binding of GP autoantibodies to autoantigen in vivo.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis and Expression of Mutated NC1 Chimera**—Eight chimeric constructs with individual $a3(IV)$ to $a1(IV)$ amino acid substitutions in the $E_a$ epitope region, designated M1–M8 (see Fig. 2), were constructed using the GeneEditor™ in vitro site-directed mutagenesis system (Promega, Madison, WI) according to the manufacturer’s protocol. Substitutions from $a3(IV)$ to $a1(IV)$ amino acids were introduced by specific primers (Table I). The previously described C26 construct (10), containing the GP epitope regions $E_A$ and $E_B$ with the $a3(IV)$ NC1 domain substituted into the $a1(IV)$ NC1 domain scaffolding, was used as a template. The clones thus obtained were digested with Nhe I and SacII restriction enzymes (New England Biolabs Inc., Beverly, MA) and subcloned into the pRcX expression vector (10, 12), which contains the BM-40 signal peptide and the FLAG peptide (DYK-
DDDDK) sequences downstream of the cytomegalovirus promoter. All constructs were sequenced to confirm the substitutions.

Human embryonic kidney 293 cells (ATCC 1573 CRL) were transfected with ~5 μg of plasmid DNA using the calcium phosphate precipitation method, and G418-resistant cells were screened for expression of recombinant protein from the culture medium by Western blotting with anti-FLAG monoclonal antibody M2 (Sigma) as previously described (10). Recombinant proteins were purified by affinity chromatography on an anti-FLAG M2 affinity column (Sigma) according to the manufacturer’s instructions. The concentration of the purified NC1 domains and chimeras was determined spectrophotometrically using 1.6 A_280 = 1 mg/ml (10).

Monoclonal Antibodies—Several monoclonal antibodies to the α3(IV) NC1 domain were used, the epitopes of which have been previously localized (7). Mab3 was purchased from Wieslab AB (Lund, Sweden), M3/1, raised against an amino-terminal synthetic peptide of the human α3(IV) NC1 domain, and monoclonal antibody 175, raised against randomly folded recombinant human α3(IV) NC1 domain expressed in Escherichia coli, were previously described (13).

GP Patient Sera—The serum or the plasmapheresis fluid from 13 patients diagnosed with Goodpasture disease (GP1–13) was used. The titer of GP antibodies was measured by ELISA in microtiter plates coated with α3(IV) NC1 domain (100 ng/well); and for further analyses, sera were appropriately diluted to yield approximately equal reactivity. The relative autoantibody reactivity to the E_A and E_B regions was measured by ELISA using the C2 and C6 chimeras, respectively (10). GP sera that reacted predominantly with E_A (GP_A autoantibodies) were used without further purification. GP sera that showed significant reactivity against the E_B region required further purification by absorption of GP_A antibodies to a column with immobilized C6 as previously described (7). The unbound fraction, consisting of GP_A antibodies, was used in the subsequent immunoassays for mapping the epitope location. The bound GP_B fraction was eluted from the C6 affinity column with 3 M guanidinium chloride and used as a control to establish the correct folding of the mutated chimeras.

Western Blots—The proteins (300 ng), separated by SDS-10% polyacrylamide gel electrophoresis under nonreducing conditions, were transferred onto nitrocellulose membranes and immunoblotted with anti-FLAG and Mab3 monoclonal antibodies as well as with GP sera as previously described (10).

Direct and Inhibition Immunoassays—The α1(IV) NC1 domain and chimeras C6 (negative controls), chimera C26 and the α3(IV) NC1 domain (positive controls), and mutated chimeras M1–M8 were coated in duplicates onto Maxisorp™ ELISA plates (Nunc) at 100 ng/well in 50 mM carbonate buffer, pH 9.6. Binding of GP autoantibodies or Mab3 was determined by direct ELISA as previously described (7). Detection was performed with alkaline phosphatase-conjugated secondary antibodies followed by p-nitrophenol phosphate, and color development was monitored at 410 nm with a Dynatech MR4000 plate reader. Each serum was analyzed at least four times, with the median of the results being taken as the serum’s representative value. For inhibition ELISA, the GP sera or purified GP antibody fractions were incubated overnight at room temperature with various amounts of recombinant NC1 domains or chimeras prior to addition to plates coated with the C26 chimera.

Statistical Analysis—The relative optical density of the mutated protein chimeras with respect to the binding of GP autoantibodies was calculated as the ratio of the absorption upon ELISA of each mutated chimera to that of the template C26 chimera. Results are reported as the mean ± S.D. of the serum’s median values. Statistics were calculated using the SPSS software package (Version 9.0). The overall significance of differences in relative binding for M1–M8 was analyzed by repeated measures-analysis of variance. Reduced binding to individual chimeras, relative to the template C26 chimera, was tested by one-sample t tests. Possible serum subgroups based on nonparallel binding profiles for M1–M8 were revealed by hierarchical cluster analysis and followed up with independent-sample t tests and Pearson correlations to identify the specific chimeras responsible for significantly divergent profiles. All statistical tests were two-tailed. Significance was inferred when p was <0.05.

RESULTS

Homolog-scanning Mutagenesis of the Immunodominant GP Epitope Region E_A—A 15-residue region of α3(IV) NC1, designated E_A (residues 17–31), was sufficient to confer reactivity for the immunodominant population of GP autoantibodies (GP_A) when substituted into the non-immunoreactive scaffold of the α1(IV) NC1 domain (7, 10). Thus, the E_A region encompasses the autoimmune site for GP_A antibodies. The E_A region also contains residues that constitute the epitope for the Mab3 monoclonal antibody, along with other critical residues from the E_B region (7). Although GP_A and Mab3 antibodies bind to the same E_A region, their respective epitopes are distinct because of dissimilar accessibility for antibody binding in the NC1 hexamer complex; this suggests that the E_A region comprises buried (or sterically hindered) residues that compose the GP_A autoimmune site as well as surface-exposed residues that compose the Mab3 epitope (7). Here, homolog-scanning mutagenesis was used to identify which of the eight α3-specific residues within the E_A region constitute the epitopes for GP_A and Mab3 antibodies.

The C2-6 chimera, which contains the E_A and E_B regions of...
the α3(IV) NC1 domain in a scaffold of the α1(IV) NC1 domain (Fig. 2, top), was used as a template for mutagenesis. The E\(_B\) region was included in the chimera because it, along with the E\(_A\) region, is required for binding of the Mab3 antibody (7). Moreover, the E\(_B\) region encompasses residues that constitute the conformational epitope for GP\(_B\) autoantibodies. Thus, the binding of GP\(_B\) antibodies can serve as a control for assessment of overall conformation of proteins mutated in the E\(_A\) region (see below). In this study, each of the eight α3-specific residues of the E\(_A\) region was substituted with the corresponding α1 residue. The mutated chimeras, designated M1–M8 (Fig. 2, bottom), were then analyzed for their ability to bind GP\(_A\) and Mab3 antibodies as well as the control GP\(_B\) antibodies.

Expression and Correct Folding of the M1–M8 Mutated Chimeras—The mutated chimeras were expressed in human embryonic kidney 293 cells. After purification from the culture medium by affinity chromatography on an anti-FLAG column, the M1–M8 mutants appeared as a single band on SDS-polyacrylamide gel electrophoresis under nonreducing conditions. Moreover, the E\(_B\) region was included in the chimera because it, along with the E\(_A\) region, is required for binding of the Mab3 antibody (7). The EB region was included in the chimera because it, along with the EA region, is required for binding of the Mab3 antibody (7). Moreover, the EB region encompasses residues that constitute the conformational epitope for GP\(_B\) autoantibodies. Thus, the binding of GP\(_B\) antibodies can serve as a control for assessment of overall conformation of proteins mutated in the EA region (see below). In this study, each of the eight α3-specific residues of the EA region was substituted with the corresponding α1 residue. The mutated chimeras, designated M1–M8 (Fig. 2, bottom), were then analyzed for their ability to bind GP\(_A\) and Mab3 antibodies as well as the control GP\(_B\) antibodies.

Expression and Correct Folding of the M1–M8 Mutated Chimeras—The mutated chimeras were expressed in human embryonic kidney 293 cells. After purification from the culture medium by affinity chromatography on an anti-FLAG column, the M1–M8 mutants appeared as a single band on SDS-polyacrylamide gel at the predicted molecular mass of ~25 kDa (Fig. 3A) and showed reactivity with anti-FLAG antibodies on Western blots (Fig. 3B). All mutated chimeras reacted with GP\(_B\) antibodies as well as or better than the template C26 chimera (Fig. 3C), indicating that the mutations did not cause protein misfolding. Moreover, certain chimeras reacted with GP\(_A\) antibodies, but not with Mab3 antibodies and vice versa (as described in detail below), further establishing that the loss of reactivity after mutagenesis was due to removal of a critical binding site and not to protein misfolding. The epitopes of GP\(_A\), GP\(_B\), and Mab3 antibodies are conformational and present only in correctly folded protein, as shown by the contrast between the recombinant α3(IV) NC1 domain expressed in human embryonic kidney 293 cells, which is correctly folded, and that expressed in E. coli, which is misfolded (10). The misfolded protein did not react with GP\(_A\), GP\(_B\), and Mab3 antibodies,
confirmed by a cluster analysis, which revealed two independ-
ences among the binding profiles of individual sera. This was
with GPA autoantibodies in inhibition ELISA.

To determine which amino acids of the EA region are
critical for the GP A autoepitope, the reactivity of the M1–M8 mutants with Mab3
was determined by Western blotting and ELISA (Fig. 6). Three of the eight mutants, M4, M7, and M8, showed no reactivity by
Western blotting and significantly decreased reactivity by
ELISA (by 97, 85, and 62%, respectively, relative to the reac-
tivity of the C2-z6 chimera; \( r = -0.766; p = 0.002 \)) (Fig. 4B). The reduced reactivity of M2, M3, M6, and M7 was confirmed by inhibition ELISA
(Fig. 5). These findings indicate that Ala18, Ile19, Val27, and Pro28 constitute the critical residues of the GP A autoepitope.

**Immunoreactivity of Mutants M1–M8 with Mab3**—To determine which amino acids in the EA region are critical for the
Mab3 epitope, the reactivity of the M1–M8 mutants with Mab3
was determined by Western blotting and ELISA (Fig. 5). The eight mutants, M4, M7, and M8, showed no reactivity by
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(Fig. 5). These findings indicate that Ala18, Ile19, Val27, and Pro28 constitute the critical residues of the GP A autoepitope.

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Although it reacted with monoclonal antibodies that recognize linear epitopes, M3/1 and 175 (Fig. 3D).

**Immunoreactivity of Mutants M1–M8 with GP A Autoantibodies**—To determine which amino acids of the EA region are critical for the GP A autoepitope, the reactivity of the M1–M8 chimeras with sera from 13 GP patients was analyzed by
ELISA (Fig. 4). To allow comparison of data across multiple experiments, the reactivity of the mutants was expressed relative to that of the template C2-z6 chimera, which constitutes the positive control because it contains the complete EA regions of a3 along with the E\(_\alpha\) region. Chimera C6, containing the E\(_\beta\) but not the E\(_\alpha\) region of the a3(IV) NC1 domain, was used as a
negative control. At least four measurements were performed for each serum. To exclude the interference of E\(_\beta\) in the binding studies, E\(_\beta\)-depleted GP sera and GP sera with little or no E\(_\beta\) reactivity were used.

For the overall group of 13 sera (Fig. 4A), the binding profile
for mutated chimeras was characterized by large reductions for
M2 (57%; \( p < 0.001 \)), M6 (44%; \( p < 0.001 \)), and M7 (67%; \( p < 0.001 \)) and a small reduction in binding for M1 (16%; \( p = 0.035 \)). The notably large variability in binding associated with
M3 (Fig. 4, A and B) suggested potentially important differences among the binding profiles of individual sera. This was
confirmed by a cluster analysis, which revealed two independent
serum groups (\( p = 0.008 \)). The eight sera in group A (Fig.
4C) had large reductions in reactivity with the M2, M6, and M7
mutants (by 56, 57, and 58%, respectively; \( p < 0.001 \)). Corrected
for reactivity of the negative control (the C6 chimera),
the respective reductions with M2, M6, and M7 were 83, 84, and 85%. In contrast, the five sera in group B (Fig. 4D) had large reductions in reactivity with mutants M2 (60%), M3 (70%), and M7 (81%) (\( p < 0.001 \)), along with small reductions with M1 (30%) and M6 (23%) (\( p < 0.05 \)). Corrected for the reactivity of the negative control, in group B, the respective
reductions with M2, M3, and M7 were 75, 85, and 96%. Group
A differed significantly from group B regarding M3, M6, and
M7 (\( p < 0.01 \)). That M3 and M6 were singularly responsible for
distinguishing the serum subgroups was shown by the large
negative correlation between M3 and M6 in the group of 13
sera (\( r = -0.766; p = 0.002 \)) (Fig. 4B). The reduced reactivity
of M2, M3, M6, and M7 was confirmed by inhibition ELISA
(Fig. 5). These findings indicate that Ala18, Ile19, Val27, and Pro28 constitute the critical residues of the GP A autoepitope.

**Immunoreactivity of Mutants M1–M8 with Mab3**—To determine which amino acids of the EA region are critical for the
Mab3 epitope, the reactivity of the M1–M8 mutants with Mab3
was determined by Western blotting and ELISA (Fig. 6). Three of the eight mutants, M4, M7, and M8, showed no reactivity by
Western blotting and significantly decreased reactivity by
ELISA (by 97, 85, and 62%, respectively, relative to the reac-
tivity of the C2-z6 chimera; \( r < 0.005 \)). This indicates that Ser21, Pro28, and Ser31 are critical residues for the Mab3 epitope.
With the exception of Pro28, this subset of amino acids is
different from that composing the GP A autoepitope, confirming
the supposition that the Mab3 and GP A epitopes are different,
but colocalized within the 15-residue EA region. Consistent
with the location of the Mab3 epitope on the surface of the NC1
hexamer, the two serine residues important for the binding of
Mab3 are hydrophilic and thus predicted to be solvent-accessible
in folded proteins.

This result also provided additional evidence that the mu-
tated chimeras were correctly folded, all showing reactivity with
GP B and either GP A or Mab3 antibodies, which bind to
conformational epitopes. Mutation of Pro28 abolished the inter-
action with both Mab3 and GP A, suggesting that this proline
may be shared by both epitopes. Alternatively, Pro28 could play a
structural role, being required for the correct conformation of
the EA region within the a3(IV) NC1 domain. The mutation
P28I did not seem to affect the overall secondary structure of
the NC1 domain because the far-UV circular dichroism spec-
trum of the M7 mutant was similar to that of the parent C2-z6
chimera and to that of the recombinant a3(IV) NC1 domain
(data not shown). Moreover, if the P28I mutation had any effect
on the conformation, this would be limited to the EA region, as
binding of GP B autoantibodies to a neighboring E\(_\beta\) epitope
was not affected.

**DISCUSSION**

The inaccessibility of the GP A autoepitope, contrasting with
the full accessibility of the Mab3 epitope, afforded an exper-
imental strategy to identify critical residues of these conforma-
tional epitopes and to explore the molecular basis of the cryptic
nature of the GP A autoepitope. To this end, the 15-residue EA
region of the a3(IV) NC1 domain was mutated at eight a3-
specific residues, and the resulting chimeras (M1–M8) were
assessed for their ability to bind GP A and Mab3 antibodies. The
M2 (A18D), M3 (I19D), M6 (V27K), and M7 (P28I) chimeras
had greatly decreased binding to GP A antibodies, whereas the
M4 (S21Q), M7 (P28I), and M8 (S31H) chimeras had greatly
decreased binding to Mab3 antibodies. The loss of binding of
GP A antibodies to one set of chimeras and of Mab3 to another,
along with binding of GP A antibodies to all chimeras, indicated
that the overall conformation of the chimeras did not differ
from that of the control (template) C2-z6 chimera. Hence, a
decrease in antibody binding to a specific chimera was due to

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**Fig. 5. Immunoreactivity of the M1–M8 mutated chimeras with GP A autoantibodies in inhibition ELISA.** Appropriately diluted GP sera were incubated with mutated (M1–M8) and control NC1 chimeras at concentrations varying between 0.01 and 10 \( \mu \)g/ml. The binding of sera containing inhibitor to plates coated with the C2-z6 chimera (100 ng/well) was determined by ELISA and compared with the binding in the absence of inhibitor (considered as 100%). The data are shown for one GP serum from group A (A) and another serum from group B (B).
removal of a critical epitope residue rather than to misfolding and/or mispairing of disulfide bonds. Thus, the binding profiles indicate that Ala18 and Pro28, along with either Ile19 in certain GP patients or Val27 in other GP patients, are critical for the GPA autoepitope, whereas Ser21, Ser31, and Pro28 are critical for the Mab3 epitope, as depicted in Fig. 7.

Interestingly, three of the four GPA residues (Ala18, Ile19, and Val27) are hydrophobic (14, 15) and have a high propensity to be buried (16). This suggests that the cryptic nature of the GPA autoepitope is because one or more GPA residues participate in hydrophobic interactions with other NC1 domains in the hexamer complex, thus burying the epitope and rendering it inaccessible for binding of autoantibodies (Fig. 7). In contrast, the Mab3 epitope is accessible because it contains critical residues that have a high propensity to be located on the hexamer surface (16): two hydrophilic serine residues and one proline residue, which is most often found in β-turns and other loops on the protein surface. Interestingly, Pro28 is critical for both GPA and Mab3 epitopes; thus, it may be important in defining the conformation of the EA region on the α3(IV) NC1 domain.

The hydrophobic character of the GPA residues represents a departure from the conventional features of epitopes, which consist predominately of charged or polar residues and tend to be located in flexible turns or loops on protein surfaces (17, 18). Based on these features, numerous empirical methods have been developed that successfully predict the major antigenic sites of native proteins (19). The α3(IV) NC1 sequence was analyzed with the program Epiplot, which calculates and plots flexibility, hydrophilicity, and antigenicity profiles using 13 different scales, chosen as those yielding the best predictions on proteins whose antigenic structures are known (20). None of

Fig. 6. Reactivity of the M1–M8 mutants with the Mab3 monoclonal antibody. The binding of Mab3 to the M1–M8 mutated chimeras was measured by Western blotting (A) and by direct ELISA (B) and was compared with the binding of Mab3 to the template C26 chimeras. Significant reduction in reactivity (p < 0.001) was observed for mutants M4, M7, and M8 (*).

FIG. 7. Schematic representation of the GPA autotepitope, highlighting quaternary interactions in the NC1 hexamer as the molecular basis of its cryptic nature. Within the EA region of the α3(IV) NC1 domain, hydrophobic residues Ala18, Ile19, Val27, and Pro28 are critical for the GPA epitope (diagonal lines). One or more of these GPA residues are buried within the NC1 hexamer by hydrophobic interactions with residues of other NC1 domains, rendering the epitope cryptic and inaccessible for antibody binding (left). Upon hexamer dissociation, the buried residues are exposed and become accessible for binding (right). In contrast, hydrophilic residues Ser21 and Ser31 as well as Pro28 are critical for the Mab3 epitope (solid black), which also includes some residues from the EA region. These residues are exposed on the surface of the NC1 hexamers and monomers, thus accessible for Mab3 binding. Pro28 is critical for both GPA and Mab3 epitopes, and it is possibly important for the native conformation of the EA region.
these methods predicted the location of the GP A autoepitope or the Mab3 epitope, which reside within the amphipathic EA region of the α3(IV) NC1 domain, as illustrated by the popular Hopp-Woods hydrophilicity plot in Fig. 8 (upper) (21). Thus, other factors must govern the immunogenicity of the EA region, eliciting the production of autoantibodies to GP A residues on one hand and of antibodies to Mab3 on the other.

In particular, the lack of immune tolerance to the GP A epitope may be explained by its cryptic nature, which renders it an immunologically privileged site. To avoid autoreactivity, the B cell clones directed to self-antigens are edited out early in development, establishing self-tolerance. Because the GP A epitope is buried in the NC1 hexamer complex under normal physiological conditions, it is sequestered from the immune system. Therefore, if pathogenic factors induce hexamer disassociation, the newly exposed GP A residues would then be perceived as “foreign” by the immune system, eliciting an autoimmune response. What factors trigger this process in vivo remain unclear. Hydrocarbons or viral infections have been suggested as causative agents (22). A recent study provides evidence that reactive oxygen species may act as the physiological mediator for epitope exposure (23).

Certain of the GP A residues identified herein differ from those found in two recent studies by Wieslander and co-workers (11, 24), who did not identify the overall hydrophobic character of the epitope. The first study qualitatively investigated the role of 14 α3(IV) NC1 domain residues, including six of the eight residues in the EA region (11). Similar to our findings, Ile18 and Pro28, but not Thr17, were found important for binding. However, Ala18 was reported to be not essential, and the role of Glu24 and Val27 was not addressed. In addition, Ser21 and Ser23 were reported to be critical for binding GP antibodies, whereas we found these residues to be important only for the binding of the Mab3 antibody. Since a quantitative data analysis was not reported in that study, it is difficult to directly compare their findings with ours. A very recent paper from the same group (24) quantitatively analyzed the role of four α3 residues in the EA region and found Val27 to be critical for GP antibody binding (similar to our findings), whereas Thr17, Ala18, and Glu24 had a moderate effect. Some of the discrepancies may be explained by the different α1α3 chimeras used as template for the homolog-scanning mutagenesis. Their template chimera contained only the EA region, whereas ours contained both the EA and EB regions of the α3(IV) NC1 domain. Because the EA and EB regions are in close proximity in the natively folded α3(IV) NC1 domain, mutants M1–M8 used in our work are likely to reproduce the native GP epitopes more closely. In addition, our study relied on a mutagenesis strategy that allowed (a) a verification of the conformation of the mutants by using three different antibodies that recognize conformational epitopes and (b) a comparison between the binding of GP A and Mab3 antibodies, a priori inferred to bind to different residues in the EA region.

The identification of the critical residues of the GP A epitope allows the determination of the structural features that selectively target GP A antibodies to the α3(IV) NC1 domain, among the six homologous NC1 domains of type IV collagen. As revealed by the comparison in Fig. 8 (lower), all four GP A residues (Ala18, Ile19, Val27, and Pro28) occur at the respective position in the α3 sequence only. Intriguingly, three of the GP A residues are hydrophobic in the α3 sequence, but the homologous α1 residues are hydrophilic, charged residues (Asp, Asp, and Lys, respectively). Analysis of data from experimentally determined antigenic sites on proteins has revealed that hydrophobic residues are more likely to be a part of antigenic sites, if they occur on the surface of a protein (25), as GP A residues do in the α3(IV) NC1 monomer. No other chain besides α3(IV) had a representation of three hydrophobic residues at positions 18, 19, and 27. Moreover, Pro28 occurs only in α3, but not in any of the other five NC1 domains. Therefore, three hydrophobic residues at positions 18, 19, and 27, together with a proline at position 28, in a distinct conformation distinguish α3 among the six NC1 domains, conferring binding of GP A antibodies selectively to the α3(IV) NC1 domain.

The EA region of the α3(IV) NC1 domain emerges as a prime candidate for a molecular recognition site that specifies the chain-specific assembly of the α3α4α5 network of type IV collagen. Recently, we showed that the NC1 monomers contain recognition sequences for selection of chains and protomers that are sufficient to encode the specificity of assembly of the α1α2 and α3α4α5(IV) networks of the glomerular basement membrane (26), but their identity is unknown. That the EA region is a site of interaction between α3 and the other NC1 domains in the α3α4α5 hexamer is deduced from its cryptic nature of the GP A epitope. That the EA region may also be responsible for the specificity of interaction is suggested by the high sequence divergence of this region among the six NC1 domains (27). The EA region is also distinguished by the highest number of non-conservative amino acid substitutions. Hence, the pattern of hydrophobic and hydrophilic residues within the EA region is unique for each of the six NC1 domains and is likely to confer chain-specific conformations and interactions. In particular, the EA region of all six NC1 domains may

![Structural features of the EA region](http://www.jbc.org/)
contribute to the discriminatory interactions that result in specific assembly of chain-specific networks of type IV collagen.

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