The Goodpasture Autoantigen

MAPPING THE MAJOR CONFORMATIONAL EPITOPE(S) OF α3(IV) COLLAGEN TO RESIDUES 17–31 AND 127–141 OF THE NC1 DOMAIN∗

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The Goodpasture (GP) autoantigen has been identified as the α3(IV) collagen chain, one of six homologous chains designated α1–α6 that comprise type IV collagen (Hudson, B. G., Reeder, S. T., and Tryggvason, K. (1993) J. Biol. Chem. 268, 26033–26038). In this study, chimeric proteins were used to map the location of the major conformational, disulfide bond-dependent GP autoepitope(s) that has been previously localized to the non-collagenous (NC1) domain of α3(IV) chain. Fourteen α1/α3 NC1 chimeras were constructed by substituting one or more short sequences of α3(IV)NC1 at the corresponding positions in the non-immunoreactive α1(IV)-NC1 domain and expressed in mammalian cells for proper folding. The interaction between the chimeras and eight GP sera was assessed by both direct and inhibition enzyme-linked immunosorbent assay. Two chimeras, C2 containing residues 17–31 of α3(IV)NC1 and C6 containing residues 127–141 of α3(IV)NC1, bound autoantibodies, as did combination chimeras containing these regions. The epitope(s) that encompasses these sequences is immunodominant, showing strong reactivity with all GP sera and accounting for 50–90% of the autoantibody reactivity toward α3(IV)NC1. The conformational nature of the epitope(s) in the C2 and C6 chimeras was established by reduction of the disulfide bonds and by PEPSCAN analysis of overlapping 12-mer peptides derived from α1- and α3(IV)NC1 sequences. The amino acid sequences 17–31 and 127–141 in α3(IV)NC1 have thus been shown to contain the critical residues of one or two disulfide bond-dependent conformational autoepitopes that bind GP autoantibodies.

Goodpasture (GP) autoimmune disease is characterized by pulmonary hemorrhage and/or rapidly progressing glomerulonephritis (1). Tissue injury is mediated by anti-basement membrane antibodies that bind alveolar and glomerular basement membranes. The target autoantigen of basement membranes has been identified as the α3(IV) collagen chain, one of six homologous chains designated α1–α6 that comprise type IV collagen (2). In the glomerular basement membrane, the α3(IV) chain exists in a supramolecular network along with the α4(IV) and α5(IV) chains (3). The α3(IV) chain is composed of a long collagenous domain of 1410 amino acids and a non-collagenous (NC1) domain of 232 residues at the carboxyl terminus (4). The GP autoepitope(s) has been localized to the NC1 domain of the α3(IV) chain (5, 6). Antibodies that bind to the NC1 domain of other α(IV) chains may be found in some Goodpasture patients (7, 8), but they only account for about 10% of autoreactivity (9). The autoepitope(s) in the α3(IV)NC1 domain appears to be conformational, because reduction of disulfide bonds abolishes most of the binding (9–11). The identification of the precise amino acid residues that constitute this epitope(s) is important for understanding the etiology and pathogenesis of the GP disease and for the development of diagnostic and therapeutic agents. Several groups have attempted to map the location of the autoepitope(s) by using short linear peptides (9, 11–14) or by site-directed mutagenesis of the α3(IV)NC1 domain expressed in Escherichia coli (15). Although linear sequences have been identified that bind GP antibodies, these findings are at variance with each other. Moreover, prior studies have not addressed whether these linear sequences constitute the major conformational, disulfide bond-dependent epitope(s).

The aim of this study was to identify the α3(IV)NC1 amino acid sequences that form the thus far elusive conformational GP epitope(s). To circumvent the limitations of previous approaches, we pursued an epitope mapping strategy based on chimeric proteins. This approach has been specifically developed and successfully used to map conformational epitopes (16) or autoepitopes (17). We hypothesized that the α3(IV)NC1 regions most likely to form the autoepitope(s) are those most divergent from the other homologous α(IV) chains. A series of chimeric α1/α3(IV)NC1 domains were constructed in which these candidate α3(IV)NC1 sequences replaced the corresponding sequences in the non-immunoreactive α1(IV)NC1. The chimeras were expressed in mammalian cells for correct protein folding and disulfide bond formation. We report that two specific sequences, α3(IV)NC1 residues 17–31 and 127–141, contain the critical residues of one or two disulfide bond-dependent conformational GP autoantibodies within the α3(IV)NC1 domain.

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§ The abbreviations used are: GP, Goodpasture; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; NC1, the non-collagenous domain of type IV collagen; PCR, polymerase-chain reaction; CMV, cytomegalovirus; Eα and Eβ, α3(IV)NC1 residues 17–31 and 127–141, respectively.

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EXPERIMENTAL PROCEDURES

cDNA Manipulation and Chimera Construction—A suitable vector (Fig. 1) for the expression of recombinant proteins was based on pRC/AC7, a derivative of pReCMV (Invitrogen) that contained an expression cassette consisting of the BM-40 5′-untranslated region, BM-40 signal peptide, and an α3 type VI collagen insert (18). By using a two-step inverse PCR with the appropriate primers (Table I), the original insert was replaced by a FLAG sequence underlined. The resulting vector (pRe-X) was used for the construction of the chimeras (Fig. 1, middle). After cleavage of the signal peptide, secreted proteins would contain at the amino terminus a 14-residue fusion sequence (AFLADYKDDDDDKLA) that included the FLAG peptide (underlined) used for affinity purification.

The cDNA for the human α1(IV)NC1 domain was amplified from a human kidney cDNA library (Marathon-Ready, CLONTECH) by PCR using KlenTaq polymerase (CLONTECH) and subcloned into pCR®II vector by using a TA cloning kit (Invitrogen). The inserts with the NC1 domain were subcloned into pRC-X. The resulting pRC-X vector was subcloned into the appropriate restriction enzyme sites (Nhe, ClaI, HpaI, and SacII) and used in the PCR reactions for its low error rates. Restriction enzymes and ligase were purchased from New England Biolabs. The correct sequence of each construct was verified by sequencing.

The principle of the inverse PCR approach that was used for chimeras C2, C3, C5, and C6 is depicted in the dashed circle in Fig. 1 (top). The primers (Table I) were designed in a back-to-back orientation, each containing (in 3′ to 5′ order) residues complementary to the α1(IV)NC1 template, residues complementary to a part of the replacement α3(IV)NC1 sequence, and the recognition site of the inward-cutting BbsI restriction enzyme (GAAGAC(N)2/6). PCR yielded 6.3-kilobase pair amplicons that comprised the whole vector and insert. Digestion with BbsI removed the recognition site and created complementary ends inside the inserted α3(IV) sequence, and then ligation produced a circular expression vector containing a chimeric α1/α3(IV)NC1 insert with no extraneous sequence.

Construction of C1 and C4 chimeras was based on a regular PCR strategy using pCR/α1 as a template and introducing α3 sequences by primers at the 5′ and 3′ ends of the NC1 insert, respectively. The PCR products were digested with restriction enzymes (Table I) and subcloned into the pRC-X vector for expression. The construction of C7 chimera followed a similar scheme, requiring C1 as template. In order to construct chimera C8, two collagenous Gly-X-Y triplets of the α1(IV) chain had first to be added to the 5′ end of the α1(IV)NC1 sequence. An α1(IV)NC1 + Gly-X-Y insert was amplified from a pCR/α1 template, digested with NheI/PvuI, and ligated into a C3-4 vector preparation cut with the same enzymes. Inverse PCR with this template generated chimera C8.

Six combination chimeras were also constructed as follows: C1-2, C1-4, C2-6, C3-5, C1-2-5, and C7-8. C1-2 chimera was generated using primers for the C1 construct and C2 as template, digested with NheI and ClaI, and then subcloned into the pRC-X vector. The remaining chimeras were generated by subcloning the chimeric insert region of one chimera into a vector preparation of another chimera digested with the same restriction enzymes. C1-4 required subcloning of a NheI/PvuMI C1 insert fragment into the C4 vector; likewise, C2-6 required an ApaI C6 insert in the C2 vector; C3-5 required a PvuMI/SacII C3 insert in the C5 vector; C1-2-5 required an ApaI C5 insert in the C1-2

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**Table I**

| Product | Primer sequences (5′ → 3′) | Enzyme |
|---------|---------------------------|--------|
| Vector  | A1<sup>a</sup> | GACGCTACGGCGCCCTGAGATCTGACTAGAGGGCC | NheI |
| Intermediate | B1<sup>a</sup> | GCGGCTACGGCGCCCTGAGATCTGACTAGAGGGCC | NheI |
| pRC-X   | A2 | AGCTCTAGATCGATCTGAGTCGTCCTTGTAGTGGGGCTGCCAGAGCCCT | BxbI |
| α1(IV)NC1 | A3 | GCTAGACATCTGGTAGACAGCCGCTCC | SacII |
| C2     | A4 | AGTGGAAAGACGGGGACAGTGGCCTCTCATTGGTTGGTGTGTTGCT | BbsI |
| C3     | A5 | GAGCGAGAACAAAGATTTGCAAAAGCTATATTTTCCCCGCTTGCAAGG | BbsI |
| C4     | A6 | CATGGAAAGACGGGGACAGTGGCCTCTCATTGGTTGGTGTGTTGCT | BbsI |
| C5     | A7 | AGTGGAAAGACGGGGACAGTGGCCTCTCATTGGTTGGTGTGTTGCT | BbsI |
| C6     | A8 | AATGTCAGAACCCTGGAACAGAGGCTTCTCTGTGAGG | NheI |
| C7     | A9 | GACATCGTCTGAGTCTGACGTCCTTATAT | ClaI |
| C8     | A10 | ATATGCTATCTGGTTTGAAGAAAACGGGAGACAGTGGTGTGTTGCTGAGG | NheI |
| α1(IV)NC1 + Gly-X-Y | B11 | GACATCGTCTGAGTCTGACGTCCTTATAT | ClaI |
| +Gly-X-Y | B12 | ATATGCTATCTGGTTTGAAGAAAACGGGAGACAGTGGTGTGTTGCTGAGG | NheI |
| C1-2    | B13 | GACATCGTCTGAGTCTGACGTCCTTATAT | ClaI |

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<sup>a</sup> Forward primer; B, reverse primer.<br><sup>b</sup> FLAG sequence underlined.
bic acid phosphate magnesium salt (Wako). Five to ten NC1 domains were selected with 250 DNA were transfected by the calcium phosphate co-precipitation amino acids from a with m g/ml G418 (Life Technologies, Inc.). Resistant cells were screened for expression of recombinant protein by Western blot using an anti-FLAG monoclonal antibody (M2, Kodak) and expressed in human embryonic kidney 293 cells (ATCC 1573-CRL) were expressed in human kidney 293 cells yields properly folded recombinant protein; and C7-8 required a PpuMISacII C8 insert in the C7 vector.

**Protein Expression and Purification**—Recombinant a13 chimeras were expressed in human embryonic kidney 293 cells (ATCC 1573-CRL) grown in Dulbecco’s modified Eagle’s medium/F-12 medium (Sigma) supplemented with 5% fetal bovine serum (Sigma) and 50 mg/ml ascorbic acid phosphate magnesium salt (Wako). Five to ten g of plasmid DNA were transfected by the calcium phosphate co-precipitation method (19) into 70% confluent 293 cells. After 2 days, transfected cells were selected with 250 mg/ml G418 (Life Technologies, Inc.). Resistant cells were screened for expression of recombinant protein by Western blot using an anti-FLAG monoclonal antibody (M2, Kodak) and expanded for quantitative expression. The medium was collected from subconfluent cultures every 48 h, and the recombinant proteins were purified by affinity chromatography on anti-FLAG M2 affinity columns (Kodak) according to the manufacturer’s instructions. Protein solutions were concentrated by ultrafiltration (Amicon) and stored at 70 °C. The concentration of recombinant protein solutions was measured spectrophotometrically at 280 nm. An average extinction coefficient A of 1.6 was calculated from the amino acid composition of the six human a1(IV)NC1 domains (20).

Recombinant a3(IV)NC1 expressed in kidney 293 cells and E. coli was prepared as described (21, 22). Native human a13(IV)NC1 was isolated from glomerular basement membrane (23). Human kidneys unsuitable for transplantation were obtained from Midwest Organ Bank, Kansas City, KS.

**Sera**—The plasmapheresis fluid or sera from eight patients diagnosed with Goodpasture syndrome (GP1–8) were used. The titer of GP autoantibodies was measured by direct ELISA in plates (Nunc) coated with a3(IV)NC1 (100 mg/well). Relative to the GP1 serum previously described (24), GP1–4 either had a titer similar to the titer, GP5–8 had about 10-fold lower, and GP7–8 had about 50-fold lower.

**Western Blots**—SDS-polyacrylamide gel electrophoresis (25) was performed in 4–20% gradient gels, under non-reducing conditions, using 500 mg of protein per lane. For immunoblotting, the proteins (200 mg of protein/lane) were transferred to nitrocellulose membranes, reacted with GP sera (1:100) and alkaline phosphatase-conjugated goat anti-human IgG (1:1000), then stained with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.

**Direct and Inhibition Immunoassays**—MaxiSorb® polystyrene microtiter plates (Nunc, Denmark) were coated overnight at room temperature with antigen (50–200 mg/well, as shown) in 50 mM carbonate buffer, pH 9.6, and then blocked with casein or BSA. In some experiments, the antigen was reduced prior to coating by treatment with 10% β-mercaptoethanol for 5 min at 100 °C. GP sera and normal human sera (negative controls) were diluted in the incubation buffer (2% casein or 2 mg/ml BSA and 0.05% Tween 20 in Tris-buffered saline). Alkaline phosphatase-conjugated goat anti-human IgG (1:2000) was used as secondary antibody. p-Nitrophenyl phosphate (1 mg/ml) in 1× diethanolamine buffer, pH 9.8, containing 0.5 mM ZnCl2 was used as substrate, and the development of color was monitored at 410 nm in a Dynatech MR4000 plate reader. For inhibition ELISA, the GP sera were incubated overnight at room temperature with various amounts of recombinant a1(IV)NC1 domains or chimeras prior to addition to plates coated with a3(IV)NC1. The results shown are the averages of duplicate determinations.

**PEPSCAN Analysis**—Mapping of linear epitopes was performed using the “PEPSCAN” method (26). A complete set of solid phase overlapping 12-mer peptides was synthesized onto polyethylene pins following the published sequences of NC1 domains of a1(IV) (GenBankTM accession number P02462) and a3(IV) collagen (GenBankTM accession number X30033). The immunoscreening of these peptides was performed by ELISA. The pins were incubated for 1 h with GP serum (diluted 1:50) and then washed three times. The bound antibody was detected by the reaction with peroxidase-labeled secondary antibody for 30 min, followed by color development with 2,2′-azino-bis-3-ethylbenzthiazoline-sulfonic acid for another 30 min.

**RESULTS**

**Design and Expression of a11(IV)NC1 Chimeras**—In this study, a13 chimeras were used to identify the conformational epitope(s) of the GP autoantigen. This strategy relied on the high sequence homology between the NC1 domains of a1(IV) and a3(IV) and collagen (71% sequence identity and six conserved disulfide bonds), which very likely adopt similar tertiary structures (27). In the chimeras, a1(IV)NC1 acted as an inert “carrier” and provided a three-dimensional scaffold for the substituted a3(IV) sequences.

Since GP sera react preferentially with a3(IV)/NC1, but not the other a1(IV)NC1 domains, the autoepitope(s) must contain amino acids specific to a3(IV)NC1. Our recent comparative analysis of the sequences of a1(IV)NC1 domains has now permitted the identification of six putative locations of the epitope(s) as short regions (less than 15 residues) in the published sequences of NC1 domains of a1(IV) and a3(IV) (28). The epitope(s) are located in the NC1 domains, were incorporated in the C7 and C8 chimeras to emulate the proteins previously used to design and express the autoepitope (15).

The recombinant chimeric proteins were expressed in the human embryonic kidney 293 cells and isolated from the culture medium as monomers with an apparent molecular mass of about 25–30 kDa by SDS-polyacrylamide gel electrophoresis (Fig. 3a). Unlike expression in E. coli (22), expression in the human kidney 293 cells yields properly folded recombinant chimeric constructs. At the positions indicated by filled circles, sequences of amino acids from a3(IV)NC1 replaced those in the a1(IV)NC1 open circles. The disulfide bonds are represented as short lines closing the loops. The arrow indicates the junction between the collagenous and NC1 domains.

**FIG. 2. Schematic illustration of the a11/3(IV)NC1 chimeric constructs.** At the positions indicated by filled circles, sequences of amino acids from a3(IV)NC1 replaced those in the a1(IV)NC1 open circles. The disulfide bonds are represented as short lines closing the loops. The arrow indicates the junction between the collagenous and NC1 domains.
NC1 domains that are indistinguishable by FT-IR or immunassays from those prepared from native sources.\(^2\) This cell line has been successfully used to express other proteins with native folding, including basement membrane proteins nidogen (29) and laminin (30).

**Immunoreactivity of α1/α3 Chimeras with GP Sera**—The reactivity of the chimeric constructs with GP sera was analyzed by Western blotting as well as by direct and inhibition ELISA. The pattern of autoantibody binding obtained in Western blots with three sera show remarkable similarities (Fig. 3, b–d). Only two chimeras, C2 and C6 (containing residues 17–31 and 127–141 of α3(IV)NC1, respectively), reacted strongly and consistently with GP antibodies, as did combination chimeras containing one or both these regions (C1-2, C2-6, C1-2-5). Some sera showed weak reactivity with other chimeras, but this appears to be due to the cross-reactivity with the α1 backbone, because it was accompanied by comparable binding to α1(IV)NC1. Remarkably, neither C7 nor C8 chimeras bound autoantibodies.

To confirm these findings, the binding of eight GP sera to immobilized chimeras was assessed in direct ELISA (Fig. 4). Sera were diluted proportionally to their titers to allow visualization of the specificity of the low titer sera side by side with the high titer sera. In general, the pattern of reactivity observed in the Western blots was also apparent in the ELISA. All sera reacted strongly with C2 chimera (which averaged 71% of the maximal reactivity, obtained with α3(IV)NC1), C1-2 (47%), C2-6 (70%), and C1-2-5 (64%). There was more variation in the reactivity toward C6 chimera (31% of the reactivity of α3(IV)NC1), which bound significantly only five out of eight sera. All but one low titer serum (GP7) bound more to C2 than to C6 chimera. Sera that showed cross-reactivity with α1(IV)NC1 bound all chimeras, producing a higher background.

The relative reactivity of any given serum toward recombinant proteins was not influenced by the dilution of the serum. This was apparent in the titration curves shown in Fig. 5, which yielded parallel lines for various immobilized proteins. Similar results were obtained with the other sera. All sera had the highest reactivity toward α3(IV)NC1, which was closely followed by C2-6 and C2 chimeras (less than a 2-fold difference in titers). The C6 chimera titers of the sera were more variable, between 2- and 10-fold lower than the α3(IV)NC1 titers, but always higher than those of α1(IV)NC1 and α2(IV)NC1 controls.

**Immunodominance of Antibodies Binding to C2 and C6 Chimeras**—It is well established that adsorption of proteins to plastic may cause denaturation, so that the antibody binding measured in direct ELISA may actually be to the denatured antigen. To rule out such artifacts, the interaction between the GP antibodies and antigen was studied in solution by inhibition ELISA in the presence of soluble chimeras and control α1(IV)NC1 domains. The inhibition curves were determined for three GP sera and were found to be similar. Typical data for one serum are shown in Fig. 6 (top panel). The inhibitory capacity of the chimeras and the control proteins followed the same order as found in direct ELISA, α3(IV)NC1 > C2-6 > C2.

\(^2\) A. Boutaud, S. Gunwar, N. Singh, K.-O. Netzer, Y. Sado, Y. Nnomiya, M. E. Noelken, and B. G. Hudson, manuscript in preparation.
soluble antigen, 10 μg/ml (Fig. 6, bottom panel). This concentration was chosen to minimize cross-reactivity with α1(IV), while giving almost complete inhibition with α3(IV)NC1. Inhibition with C2-6 was 65 ± 13%, compared with 85 ± 7% for control α3(IV)NC1, demonstrating that this chimera contains the immunodominant autoepitope(s) of α3(IV)NC1. C2-6 chimera had a stronger effect than either C2 (46 ± 8%) or C6 chimeras (23 ± 18%). This indicates that the α3(IV)NC1 residues 17–31 (hereafter referred to as Eα) and 127–141 (hereafter referred to as Eβ) form either two separate epitopes or a single, more complete one, but it appears to rule out significant cross-reactivity between the two homologous sequences.

The data were further analyzed to estimate the fraction of autoreactivity that could be attributed specifically to the α3(IV)NC1 sequences in the chimeras. For each serum, the inhibition produced by the α1(IV)NC1 domain (which averaged 7 ± 4%) was subtracted from the total inhibition given by the chimeras to correct for the cross-reactivity due to the common scaffold, and then the results were normalized to the effect produced by α3(IV)NC1 (Table II). The effect of Eα (present in C2 chimera) was strong and consistent (on average 47%, ranging between 27 and 64%) and predominated in seven out of eight sera. In contrast, Eβ (present in C6 chimera) produced variable inhibition with different sera (on average 18%, ranging between 3 and 56%) and was predominant only in GP7. Together, as in C2-6 chimera, these sequences accounted for most inhibition of GP sera (on average 68%, ranging between 52 and 88%). Only a small fraction of GP reactivity toward α3(IV)NC1 (on average 23%, ranging between 6 and 38%) could not be accounted for by Eα, Eβ, or by cross-reactivity with α1(IV)NC1.

Conformational Nature of the Epitope(s)—It has been previously shown that the reduction of disulfide bonds in α3(IV)NC1 impairs its ability to react with GP antibodies, indicative of a conformational epitope (9–11). Quantitation of this effect with the eight sera used in the present work showed that only 6 ± 5% of the original immunoreactivity remains after reduction of α3(IV)NC1. To evaluate whether Eα and Eβ form a linear or a conformational epitope, the GP reactivity of the α1α3 chimeras was measured before and after reduction. As in α3(IV)NC1, the reduction of disulfide bonds also abolished binding of autoantibodies to the C2, C6, and C2-6 chimeras and even to α1(IV)NC1 (Fig. 7). Less than 10% of the original immunoreactivity remained in the reduced proteins, although they had the same or higher reactivity with monoclonal antibodies that do not require a conformational epitope, such as anti-FLAG (data not shown). Overall, these results demonstrate that only a small proportion of GP antibodies can recognize linear epitopes and that Eα and Eβ belong to one or two conformational GP epitopes that are disulfide bond-dependent.

Comparison of Chimera-based Epitope Mapping with Previous Approaches—to compare the chimera-based epitope mapping strategy with approaches using linear peptides (9, 11, 13, 14) and to evaluate whether the latter identify linear or conformational GP epitopes, a peptide scanning analysis was performed. A valid comparison between the chimera-based and peptide-based strategies required using the same GP sera. Two complete sets of overlapping 12-mers based on the α1(IV)- and α3(IV)NC1 sequences (Fig. 8) were therefore synthesized and analyzed by the PEPSCAN procedure (26). A previous report using 20-mer peptides to map the GP epitope has indicated nonspecific binding of GP sera to homologous α1- and α3(IV)NC1 peptides (9).

The PEPSCAN results demonstrated lack of strong specific binding and a high background, presumably due to nonspecific binding. Both α1- and α3(IV)NC1 sequences produced a num-

***Fig. 6. Inhibition ELISA of GP antibodies binding to α3(IV)NC1 domain.*** Top, inhibition of GP1 serum by soluble α1α3 chimeras. The GP1 serum, diluted 1:100, was incubated overnight with C2 (open circles), C6 (open triangles), and C2-6 (open squares) chimeras at various concentrations before the immunoassay. Recombinant α1(IV)NC1 (filled circles), α2(IV)NC1 (filled triangles), and α3(IV)NC1 (filled squares) domains were used as controls. Bottom, comparison of eight GP sera by inhibition ELISA using α1α3 chimeras and α1IVNC1 domains. Sera, diluted as described in Fig. 4, were incubated overnight with 10 μg/ml antigen. ELISA was performed in plates coated with 50 ng/well α3(IV)NC1. Individual data for the eight sera are represented by *symbols*, and their average is shown as a *horizontal line.*
number of peaks higher than two standard deviations above the median. However, the most reactive peptides (above three standard deviations) varied among the three GP sera tested. The most significant PEPSCAN peak was produced by peptides overlapping residues 94–110 of α3(IV)NC1 with the GP2 serum. Much weaker reactivity was recorded in this region with the other two sera. This region corresponds to the C6 chimera that did not interact with GP sera in direct ELISA (Fig. 4), perhaps due to the conformations of the 12-mer peptides on the pin being different from those adopted by the same amino acids in the NC1 domain. Some isolated α3-derived peptides that overlapped the E6 region produced peaks in PEPSCAN. However, the interactions were not strong enough to allow unambiguous identification of these residues as part of a GP autoepitope.

Two epitopes previously found by mutagenesis of α3(IV)NC1 expressed in E. coli (15) were not observed in C7 and C8 chimeras, made in eukaryotic cells in the present work. Recombinant α3(IV)NC1 was measured in the present study, a new strategy based on chimeric proteins to account for the contribution of cross-reactivity, and then the results were normalized to the inhibition obtained with the control α3(IV)NC1 domain (100%).

The specificity not assigned represented the amount of binding that did not interact with GP sera in direct ELISA (Fig. 4), and assigned a region being different from those adopted by the same amino acids in the NC1 domain. Some isolated α3-derived peptides that overlapped the E6 region produced peaks in PEPSCAN. However, the interactions were not strong enough to allow unambiguous identification of these residues as part of a GP autoepitope.

Two epitopes previously found by mutagenesis of α3(IV)NC1 expressed in E. coli (15) were not observed in C7 and C8 chimeras, made in eukaryotic cells in the present work. Recombinant α3(IV)NC1 was measured in the presence of soluble C2, C6, C2-6 chimeras and α3(IV)NC1 at 10 μg/ml (Fig. 6B). The inhibition produced by α1(IV)NC1 was subtracted from the values obtained with the chimeric proteins to account for the contribution of cross-reactivity, and then the results were normalized to the inhibition obtained with the control α3(IV)NC1 domain (100%).

The specificity not assigned represented the amount of binding that did not interact with GP sera in direct ELISA (Fig. 4), and accounted for 50–90% (on average 68%) of the resulting chimeras were assayed for “gain-of-function,” i.e. capacity to bind autoantibodies, in contrast with previous site-directed mutagenesis studies (15) that relied on a “loss-of-function” of the protein expressed in E. coli. The results from 14 different chimeras revealed two previously unidentified regions, designated E6 and E9 (residues 17–31 and 127–141 of α3(IV)NC1, respectively), that strongly bound autoantibodies from eight GP patients. Together, E6 and E9 accounted for 50–90% (on average 68%) of autoactivity to α3(IV)NC1.

Among the six candidate regions evaluated in this study, regions E6 and E9 clearly exhibited a distinct capacity to bind GP antibodies by Western blots, direct ELISA, and inhibition ELISA. The six regions were selected based on the following:

(a) autoantibodies preferentially bind the α3(IV)NC1 domain but not the other five homologous NC1 domains of type IV collagen; (b) therefore, regions of substantial sequence diver-
Most remarkably, EA demonstrated by loss of GP immunoreactivity of the regions that require intact disulfide bonds to bind GP antibodies, as successfully identified the critical regions of one or two immunoreactive in different studies, suggesting heterogeneity of the regions for the GP epitope. It is significant that the framework of the chimeras is instrumental for adoption of the tendency of peptide-based methods to identify sequential epitope(s). An intrinsic fine analysis is in full agreement with the broader conclusions of that study, namely that the amino-terminal third of α3(IV)NC1 accounts for most immunoreactivity with GP sera.

In summary, two specific homologous sequences in α3(IV)NC1 have been identified for the first time to be part of one or two disulfide bond-dependent, conformational and immunodominant GP autoepitopes. This finding provides new knowledge to investigate further the pathogenesis of GP disease. It has recently been shown that α3(IV)NC1 but not α1(IV)NC1 can induce experimental GP disease in mice (21).

The existence of an immunodominant GP epitope should be useful for the development of more specific therapeutic approaches, such as use of vaccines to induce tolerance or the manipulation of the idiotype network.

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A report published at the time of submission of this study (38) also used α1α3 chimeras, but larger regions of α3(IV)NC1 were swapped (the amino-terminal 54 amino acids, the carboxyl-terminal 65 amino acids, and the intervening 115 amino acids, respectively). The conformational nature of the epitopes identified was not explored. Our more fine analysis is in full agreement with the broader conclusions of that study, namely that the amino-terminal third of α3(IV)NC1 accounts for most immunoreactivity with GP sera.
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