Goodpasture Disease

CHARACTERIZATION OF A SINGLE CONFORMATIONAL EPITOPE AS THE TARGET OF PATHOGENIC AUTOANTIBODIES*

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Goodpasture disease is a prototype autoimmune disease characterized by the formation of autoantibodies against the heterotrimeric basement membrane collagen type IV, which causes a rapidly progressive glomerulonephritis. The pathogenic antibody response is directed to the non-collagenous (NC1) domain of the α3 chain of type IV collagen (α3(IV)NC1), but not to the homologous region of the α1(IV)NC1. To identify the conformation-dependent immunodominant epitope on the α3(IV)NC1, a variety of recombinant NC1 domains were constructed by replacing single residues of α3(IV) with the corresponding amino acids from the nonreactive α1(IV) chain. Replacement mutations were identified that completely destroyed the Goodpasture epitope in the α3(IV) chain. Based on the identification of these critical positions, the epitope was finally reconstructed within the frame of the α1(IV) chain. The substitution of nine discontinuous positions in the α1(IV)NC1 with amino acid residues from the α3 chain resulted in a recombinant construct that was recognized by all patients’ sera (n = 20) but by none of the sera from healthy controls (n = 10). This provides, for the first time, the molecular characterization of a single immunodominant conformational epitope recognized by pathogenic autoantibodies in a human autoimmune disease, representing the basis for the development of new epitope-specific strategies in the treatment of Goodpasture disease.

The Goodpasture syndrome fulfills all criteria of a classic autoimmune disorder in analogy to Koch’s postulate for infectious diseases. The criteria demand that an adaptive immune response to a self-antigen cause the observed pathology, as evidenced by transfer of the disease in either a natural situation (for example, from mother to child via the placenta) or an experimental disease model (via autoantigen-specific antibodies or lymphocytes) (1). In Goodpasture syndrome, the complex formation of autoantibodies with self-structures in the glomerular basement membrane causes a rapidly progressive glomerulonephritis, often accompanied by life-threatening pulmonary hemorrhage. The self-epitope is localized to the NC1 domain of the α3 chain of type IV collagen (α3(IV)NC1), a major basement membrane component of kidney glomeruli and pulmonary alveoli, thus explaining the tissue distribution of the disease. The pathogenic role of the B-cell response to this self-antigen is clearly supported not only by the transfer of disease to monkeys using kidney-bound autoantibodies from Goodpasture patients (2) but also by the therapeutic efficacy of removal of circulating antibodies by plasma exchange, as well as that of immunosuppressive treatments (3).

The name Goodpasture syndrome was given in 1958 by Stanton and Tange (4) in recognition of a patient described by Ernest Goodpasture in 1919 (5). In 1984, the antigen was identified as a new subunit of the NC1 domain of type IV collagen (6). This resulted in the cloning of the antigen (7) and the designation of the α3(IV) chain. The epitope has been characterized as a cryptotope because disintegration of the supramolecular arrangement of the basement membrane type IV collagen by denaturants leads to the exposure of critical epitope determinants, thereby enhancing the immunoreactivity with patient sera (8). In turn, chemical reduction completely abolishes immunoreactivity, indicating the involvement of disulfide bonds in the stabilization of the epitope. These features clearly indicate the involvement of a three-dimensional epitope that is normally hidden inside the intact glomerular basement membrane. Remarkably, despite an expected inter-patient variability, the ELISA1 reactivity of the patients’ autoantibodies has been successfully blocked with a monoclonal antibody (9, 10), indicating a limited epitope recognition. The carboxyterminal end of the α3(IV)NC1 domain has been suggested to harbor the Goodpasture epitope, based on a study using synthetic peptides (11). However, this study suffered from the disadvantage of using linear peptides to characterize a three-dimensional epitope; therefore, the results could not be subsequently confirmed. In turn, more recent studies have pointed out the importance of the amino-terminal region of the NC1 domain (12–14). Along this line, our group has recently shown that only autoantibodies reacting with the amino-terminal portion of the α3(IV)NC1 have a pathological impact on kidney survival (15).

Several studies aimed at identifying antibody epitopes of potential pathogenic relevance have also been performed using experimental animal models of autoimmune diseases. In contrast, little is known about epitopes in human autoimmune diseases at a molecular level, most likely due to methodological problems related to a diverse reactivity pattern of the autoantibodies. More importantly, conformational requirements of the epitopes may limit the application of linear synthetic peptides for mapping strategies. Therefore, an experimental procedure

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1 The abbreviation used is: ELISA, enzyme-linked immunosorbent assay.
was chosen that allows the expression of the antigen as a recombinant protein in a human cell line (HEK-293). This strategy enables the construction of a variety of properly folded chimeric molecules in which the α3(IV)NC1 sequence, which harbors the Goodpasture epitope, can be progressively replaced by the corresponding sequence of the homologous α1(IV)NC1, which is not recognized by the toxic autoantibodies. Indeed, the high homology between both chains, as well as the conserved disulfide bonds, enhances the likelihood for proper folding of the hybrid constructs as a prerequisite for conformation-dependent autoantibody binding.

The present study focuses on the molecular characterization of the Goodpasture epitope in the amino-terminal part of the α3(IV)NC1 domain, to which the antibody response correlates with kidney disease. For this purpose, chimeric constructs were devised in which the clinically irrelevant central and carboxyl-terminal parts of the NC1 domain were expressed as α1(IV).

**EXPERIMENTAL PROCEDURES**

**Patients and Sera**

The sera of 20 Goodpasture patients with biopsy-proven anti-glomerular basement membrane nephritis were obtained from the serum bank at the Department of Nephrology, Lund University. All patients showed crescentic glomerulonephritis with linear deposits of IgG in direct immunofluorescence. Seven of the patients also had an overt lung hemorrhage. The sera of 9 other patients with biopsy-proven anti-glomerular basement membrane nephritis were obtained from the serum bank at the Hospital for Sick Children, London, UK. For this purpose, chimeric NC1 domains (the D2–D7 constructs in Table II), the overlap region between α3(IV)NC1 and α5(III)NC1 (the D1 construct in Table II), this region was subjected to site-directed mutagenesis, five and nine codons were changed from wild type, and the selection criteria were based on the identity in the human and bovine α3(IV) sequences, as well as on concurrent, nonconvergent exchanges in the corresponding amino acid residues of the human α1(IV) and α5(IV) sequences. These 14 amino acid residues are illustrated in Fig. 1 by numbering their positions in the sequence. The point mutations were introduced in the D1 construct by the megaprimer method (20) using primers 6–20 in Table I, using the D1 construct as a template.

**DNA Constructs**

All restriction enzymes and ligase were purchased from Roche Molecular Biochemicals. The primers used are shown in Table I. The FhoI-DNA polymerase was purchased from Stratagene (La Jolla, CA). **Construction of Primary Vectors**—The primary type X-type IV collagen chimera was constructed using α3(IV)NC1 cDNA cloned in pBlue-script SK– vector (Stratagene) with a BamHI site in the 5′ end and a NotI site in the 3′ end. The full-length type X collagen cDNA in the pBluescript SK– vector was cloned between the HindIII and NotI sites. Both plasmids were cleaved with BamHI and NotI, and the α3(IV)NC1 cDNA was ligated into the internal BamHI site of type X collagen cDNA. This construct contained five unique restriction enzyme sites: a HindIII site in the 5′ end of the construct, a BamHI site in the junction between type X collagen and the type IV collagen NC1, HindIII and Xhol in the α3(IV)NC1 cDNA, and a NotI site 3′ of the coding DNA. The BamHI, HindIII, Xhol, and NotI cleavage sites were introduced into the α1(VN1) cDNA by polymerase chain reaction (Perkin-Elmer Gene-Amp 2400) using primers 1–6. Upon restriction with the appropriate enzymes, the fragments of the α1(IV)NC1 cDNA were used to replace the corresponding cassettes of the wild type α3(IV) sequence, as described previously (15). The constructs were restricted with HindIII and NotI and subcloned into a cytomegalovirus promoter-driven expression vector (pcDNA3; Invitrogen, Leek, the Netherlands). Because the major epitope region could be harbored in the amino-terminal domain of the α3(IV)NC1 (the D1 construct in Table II), this region was subjected to further mutational analysis.

**Division of the NC1 Domain**—For the construction of six α1(III) IV chimeric NC1 domains (the D2–D7 constructs in Table II), the overlap extension polymerase chain reaction technique (19) was applied using the primers 6–20 in Table I, using the D1 construct as a template.

**Replacement Mutations of Single Amino Acid Residues in the α3(IV) Sequence**—Fourteen positions were selected for mutational analysis in a chimeric construct that harbors the amino-terminal domain of the α3(IV)NC1 (from A to G in Fig. 1). The selection criteria were based on the identity in human and bovine α3(IV) sequences, as well as on concurrent, nonconvergent exchanges in the corresponding amino acid residues of the human α1(IV) and α5(IV) sequences. These 14 amino acid residues are illustrated in Fig. 1 by numbering their positions in the sequence. The point mutations were introduced in the D1 construct by the megaprimer method (20) using primers 6, 7, and 21–34.

**Replacement Mutations of Single Amino Acid Residues in the α3(IV) Sequence**—As the last step, replacement mutations were introduced into chimeric constructs comprising the entire α1(IV)NC1 domain. By site-directed mutagenesis, five and nine codons were changed from wild type α3(IV) to the corresponding α5(IV), respectively. The substitutions were introduced by an overlap extension polymerase chain reaction (19) using primers 6 and 7 with primers 35–40 and primers 6 and 7 with primers 15, 19, 20, 21, 39, and 40, respectively.

**General Handling of the Constructs**—After sequencing, all constructs were tested for the translation of a protein with the correct molecular weight using an in vitro system (Promega, Madison, WI) with [35S]cysteine and 7T RNA polymerase. Before transfection, the plasmid DNA was linearized using ScaI.
ELISA

Native type IV collagen NC1 domains were purified as described previously (10) and coated at 0.5 µg/ml. The coating efficiency of the different recombinant proteins was calibrated by their equal immuno-reactivity with the anti-collagen type X monoclonal antibody (16). Human sera were diluted 1:100, and the monoclonal antibodies were diluted 1:1000. The ELISA was performed following standard procedures (10) using alkaline phosphatase-conjugated swine anti-human IgG (Orion Diagnostica AB, Trosa, Sweden) or rabbit anti-mouse IgG (DAKO, Glostrup, Denmark) as secondary antibodies. Results were considered positive when the absorbance values exceeded the mean + 2 S.D. of 10 healthy control sera. Notably, none of the control sera was positive in any ELISA.

Inhibition ELISA

Dilutions of human sera were adjusted to give the same absorbance after 1 h in a conventional ELISA with purified native type IV collagen NC1 domains and preincubated overnight at 4 °C with the different inhibitors, i.e. recombinant or purified native proteins in concentrations ranging from 0.0025 to 25 µg/ml. The amount of IgG that reacted with surface-bound Goodpasture antigen (despite the presence of inhibitor molecules in the fluid phase) was determined after extensive washing procedures with alkaline phosphatase-conjugated secondary antibodies as described above.

SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting

One ml of the cell supernatants was precipitated with Triton X-100 and trichloroacetic acid. The precipitate was applied to SDS-polyacrylamide gel electrophoresis in 10–16% gradient gels under nonreducing conditions (21). Immunoblot experiments were performed on samples that were separated with SDS-polyacrylamide gel electrophoresis and transferred to Immobilon polyvinylidene difluoride membrane (Millipore, Saint-Quentin, France) (22).

| Table II |
| --- |
| **Construction design (see Fig. 1 for nomenclature and amino acid positions)** |
| **Name** | **Construct** | **No. of immunoreactive sera** |
| **Regional mapping (division of the NC1 domain)** | | |
| D1 | α3 from A to G then α1 | 20/20 |
| D2 | α1 from A to B then α3 to G then α1 | 20/20 |
| D3 | α1 from A to C then α3 to G then α1 | 20/20 |
| D4 | α1 from A to D then α3 to G then α1 | 0/20 |
| D5 | α3 from A to F then α1 | 20/20 |
| D6 | α3 from A to E then α1 | 4/20 |
| D7 | α3 from A to D then α1 | 0/20 |
| **Replacement mutations aimed at destroying the epitope (substitutions in the α3(IV)NC1)** | | |
| P1 | α3 from A to G then α1 substitution in position 1, D to S | 20/20 |
| P2 | α3 from A to G then α1 substitution in position 2, A to G | 20/20 |
| P3 | α3 from A to G then α1 substitution in position 3, R to H | 20/20 |
| P4 | α3 from A to G then α1 substitution in position 4, F to V | 20/20 |
| P5 | α3 from A to G then α1 substitution in position 5, A to D | 20/20 |
| P6 | α3 from A to G then α1 substitution in position 6, I to D | 7/20 |
| P7 | α3 from A to G then α1 substitution in position 7, S to Q | 5/20 |
| P8 | α3 from A to G then α1 substitution in position 8, E to S | 20/20 |
| P9 | α3 from A to G then α1 substitution in position 9, P to I | 0/20 |
| P10 | α3 from A to G then α1 substitution in position 10, S to H | 0/20 |
| P11 | α3 from A to G then α1 substitution in position 11, F to Y | 20/20 |
| P12 | α3 from A to G then α1 substitution in position 12, L to A | 20/20 |
| P13 | α3 from A to G then α1 substitution in position 13, Q to R | 4/20 |
| P14 | α3 from A to G then α1 substitution in position 14, T to S | 20/20 |
| **Reconstruction of the epitope (substitutions in the α1(IV)NC1)** | | |
| S1 | Substitutions from α1 to α3 in position 6 (D to I), 7 (Q to S), 9 (I to P), 10 (H to S), 13 (B to Q) | 0/20 |
| S2 | Substitutions from α1 to α3 in position 5 (D to A), 6 (D to I), 7 (Q to S), 8 (S to E), 9 (I to P), 10 (H to S), 13 (R to Q) and in addition I to T amino acid 35, R to V amino acid 45 | 20/20 |

* *n = 20 patients with biopsy-proven Goodpasture disease. The immunoreactivity was assessed by ELISA experiments (see “Experimental Procedures” for details).*
RESULTS

For the construction of recombinant chimeric molecules that harbor the Goodpasture epitope, the amino-terminal two-thirds of the α1 chain of type X collagen, including the leader sequence, was fused to the NC1 domain of type IV collagen (Fig. 1). The use of type X collagen in the amino-terminal part of the recombinant molecule results in a protein that is exported from the cells into the cell culture medium. Furthermore, the type X collagen part of the molecule is invariable and can therefore serve as a flag for protein detection. The epitope mapping strategy with these constructs comprised four subsets of experiments: 1) the NC1 domain was divided into three parts and separately replaced the 3(IV) sequence with the corresponding α1(IV) sequence for each part. This was done in a previous study and showed that the amino-terminal portion of the 3(IV)NC1 harbors the major Goodpasture epitope and that only antibodies against this part of the molecule correlate with kidney disease (15); 2) the immunoreactive sequence was narrowed down in the amino-terminal part of the 3(IV)NC1 to identify replacements that interfere with antibody recognition; and 3) point mutations were introduced in the corresponding α1(IV) residues in construct D1 (Table II), resulting in different chimeric molecules that harbor a single amino acid exchange (named P1–P14 in Table II). Despite slight differences in the reactivity of sera from different individuals with the recombinant proteins, two of the constructs, namely P9 and P10, remained totally negative with all patients’ sera, whereas the mutations P6, P7, and P13 at least partially abolished their reactivity.

The results on the localization of critical positions for immunoactivity by single amino acid residue substitutions were in concordance with the effect of replacement mutations of longer stretches of the α3(IV) sequence by the corresponding α1(IV) domains in the amino-terminal portion of the NC1 domain. Thus, all essential positions localize within the same 42-amino acid-long region (between position C and E in Fig. 1) that has remained confined to the 15 amino acid residues of α3(IV) from position C to E (Fig. 1).

**Amino Acid Substitutions in the α3(IV) NC1 That Disturb Autoantibody Recognition—**Comparison of the amino acid sequences for the α1(IV) and α3(IV) chains revealed 33 nonconserved residues in the amino-terminal portion of the NC1 domain from position A to G (Fig. 1). For the identification of amino acid differences of critical importance for antibody binding, the sequence comparison was extended to bovine α3(IV) and human α5(IV) sequences. This strategy was chosen because bovine α3(IV) is recognized by autoantibodies from Goodpasture patients, in contrast to the nonreactive human α5(IV) (23). Only positions with identity in the human and bovine α3(IV) sequences but with differences from the corresponding human α1(IV) and α5(IV) sequences were then selected for the introduction of replacement mutations. The criterion was fulfilled by 14 amino acid residues, the localization of which is indicated by the numbering of positions 1–14 in Fig. 1. In these positions, the α3(IV)-specific codons were replaced with the corresponding α1(IV) residues in construct D1 (Table II), resulting in different chimeric molecules that harbor a single amino acid exchange (named P1–P14 in Table II). Despite slight differences in the reactivity of sera from different individuals with the recombinant proteins, two of the constructs, namely P9 and P10, remained totally negative with all patients’ sera, whereas the mutations P6, P7, and P13 at least partially abolished their reactivity.

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Reconstruction of the Goodpasture Epitope by Site-directed Mutagenesis in the α1(IV)NC1—Based on the knowledge of positions in the α3(IV)NC1, in which a single replacement mutation can destroy immunoreactivity, we attempted the reconstruction of the Goodpasture epitope, within the frame of the nonreactive α1(IV)NC1, by site-directed mutagenesis of five and nine amino acid residues, respectively. First, the five critical residues in positions P6, P7, P9, P10, and P13 (with a negative effect of mutations on autoantibody binding) were replaced in the α1(IV)NC1 with the corresponding amino acid residues I, S, P, S, and Q from the α3(IV) sequence (construct S1 in Table II). Second, four additional nonconserved residues were substituted that are localized in the region between position C and E in Fig. 1. The resulting α1(IV)NC1 construct harbored eight amino acid substitutions from the α3(IV) sequence between position C and E in Fig. 1 and the amino acid exchange R to Q in the P13 position (construct S2 in Table II).

The first construct, S1, reacted only weakly with Goodpasture sera in immunoblotting experiments (Fig. 2); also, the mutational changes were insufficient to render the molecule reactive with patient sera in ELISA. In contrast, the second chimeric protein, S2, was recognized by all sera in both immunoblotting (Fig. 2) and ELISA, thereby indicating that the reactivity of the Goodpasture autoantibodies is directed toward this limited region in the NC1 domain of type IV collagen. Notably, in this respect, there was no difference between the epitope specificities of autoantibodies from patients with or without overt lung bleeding. In contrast to the 20 Goodpasture patients, there was no reactivity in the 10 sera of healthy humans used as a control (Fig. 3). All reactivity of the Goodpasture autoantibodies is directed toward the S2 chimeric protein, whereas the S1 construct proved insufficient for competitive binding, thus consistent with the results of a conventional ELISA shown in Table II. Below saturation, the D1 and S2 chimeric proteins bound to the Goodpasture antibodies with an affinity comparable to recombinant α3(IV)NC1. The maximal inhibitory capacity, in turn, differed slightly between D1 and S2 on one side (approximately 85%, ranging from 65% to 90%) and the complete α3(IV)NC1 (virtually 100%). Thus, as many as 85% of the autoantibodies present in the Goodpasture sera recognized an

Affinity of Goodpasture Autoantibodies to the Chimeric Constructs—The affinity of the proteins to the Goodpasture sera (n = 20) was determined by inhibition ELISA experiments using recombinant α3(IV)NC1, D1, S1, and S2 to block the binding to native NC1 domains from type IV collagen (Fig. 4). The results confirmed that the major epitope in Goodpasture syndrome is preserved in the S2 chimeric protein, whereas the S1 construct proved insufficient for competitive binding, thus
epitope that was fully contained in the critical nine amino acid residues presented in the S2 construct (Table II). Conversely, it was likewise remarkable that only 15% of the patient autoantibodies recognized unrelated structures. These observations were observed consistently in all 20 Goodpasture patients.

**DISCUSSION**

This study provides the first molecular characterization of a conformational B-cell epitope that is the univocal target of pathogenic autoantibodies in a human autoimmune disease. In Goodpasture syndrome, the anti-type IV collagen autoantibodies cause immune complex formation in the basement membranes of the kidney glomeruli and lung alveoli, thereby leading to fatal kidney disease and lung bleeding. Because the toxic autoantibodies are highly selective for the α3 chain of the heterotrimeric type IV collagen, its immunoreactive domains were subjected to a mutation analysis by replacements with the heterotrimeric type IV collagen, its immunoreactive domains residues in the amino-terminal part of the α1(IV) chain in chimeric recombinant constructs. Although the different sera showed small differences in fine specificity, this mapping strategy allowed the identification nine amino acid residues in the amino-terminal part of the α3(IV)NC1 as the recognition site of all 20 sera derived from biopsy-proven Goodpasture patients. Replacement mutations of these nine amino acid residues from the α3(IV) into the wild-type α1(IV) sequence converted the nonreactive α1(IV)NC1 into a recombinant hybrid molecule that was recognized by all patients’ sera with comparable affinity to that of the native purified Goodpasture antigen, as shown by competition ELISA. In contrast to most other autoimmune diseases, 100% of the patients with Goodpasture disease have autoantibodies against the α3(IV) chain. Furthermore, all Goodpasture patients have one major epitope. In another autoimmune disease, rheumatoid arthritis, antibodies against type II collagen are found in 30–70% of the samples (25). Furthermore, at least in animal models of collagen II-induced arthritis, there seem to be antibodies against several different epitopes (18), which complicates the epitope mapping considerably.

The identified nine amino acid residues are localized in the amino-terminal part of the α3(IV)NC1 and form a discontinuous epitope in close vicinity to the cysteine residues, which are critically involved in the folding of the NC1 domain (24) (Fig. 5). The formation of disulfide bonds between the cysteine residues stabilizes the tertiary structure in the native NC1 domain, bringing the identified amino acid residues in a reciprocal spatial relationship that is critical for antibody recognition. Conversely, it is very likely that breakage of the disulfide bonding dramatically affects the position of the critical residues, consistent with earlier results documenting the loss of immunoreactivity upon reduction of the Goodpasture antigen (8).

The epitope localization within the complex suprastructure of the basement membrane cannot be predicted at present, due to the lack of appropriate structural models. Thus, the extent to which the critical amino acid residues are normally exposed to the immune system and which mechanisms enhance the accessibility for autoimmuneprecognition in vivo remain a matter of speculation. Whereas the etiology of Goodpasture syndrome is still unknown, the restricted reactivity of autoantibodies from different patients strongly indicates an antigen-driven autoimmune process. It is therefore tantalizing to speculate that an immune response to a heterologous determinant of an infectious agent may mimic the Goodpasture epitope in the amino-terminal part of the α3(IV)NC1. The close correlation between the specificity of the autoantibody response and the clinical outcome indicates, in any case, that the epitope is part of a region with important biological function, e.g. the maintenance of basement membrane integrity. This may be disturbed by the interference of antibody binding with the homotypic interactions of the collagen molecules in the meshwork association.

The present investigation provides important structural information about the immunodominant B-cell epitope in Goodpasture syndrome. This is the first example in a human autoimmune disease of a conformation-dependent epitope, which is recognized by the pathogenic autoantibodies in all affected individuals but by none of the sera from healthy controls. Moreover, spreading of the autoantibody response to other determinants seems to be a rather limited event in Goodpasture disease. This new information may prove valuable not only for diagnostic and prognostic purposes but also for the development of therapeutic epitope-specific immunomodulatory strategies. The present study may also bear important implications for the understanding of other human autoimmune diseases.

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