Molecular Properties of the Goodpasture Epitope*

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Goodpasture disease fulfills all criteria for a classical autoimmune disease, where autoantibodies targeted against the non-collagenous domain of the α3-chain of collagen IV initiates an inflammatory destruction of the basement membrane in kidney glomeruli and lung alveoli. This leads to a rapidly progressive glomerulonephritis and severe pulmonary hemorrhage. Previous studies have indicated a limited epitope for the toxic antibodies in the N-terminal part of the non-collagenous domain. The epitope has been partially characterized by recreating the epitope in the non-reactive α1-chain by exchanging nine residues to the corresponding ones of α3. In this study we have investigated to what extent each of these amino acids contribute to the antibody binding in different patient sera. The results show that seven of the nine substitutions are enough to get an epitope that is recognized equally well as the native α3-chain by all sera from 20 clinically verified Goodpasture patients. Furthermore, the patient sera reactivity against the different recombinant chains used in the study are very similar, with some minor exceptions, strongly supporting a highly defined and restricted epitope. We are convinced that the restriction of the epitope is of significant importance for the understanding of the etiology of the disease. Thereby also making every step on the way to characterization of the epitope a crucial step on the way to specific therapy for the disease.

Goodpasture disease is known and characterized as a classic autoimmune disease. The disease is B cell and antibody mediated, with autoantibodies directed against proteins in the glomerular basement membrane and lung alveoli. When bound to self-structures in the kidney and lung, the antibodies initiate an inflammatory destruction of tissue by recruitment of complement leading to rapidly progressive glomerulonephritis often accompanied with severe and life threatening lung hemorrhage. The major self-epitope is located on the α3-NC1 domain of collagen IV. Collagen IV α3-chain has a limited distribution in the body and is only found in a few specialized basement membranes including the glomerular and alveolar basement membranes, thus explaining the specific organ involvement in Goodpasture disease. Goodpasture disease is indeed an antibody mediated disease as proven by the transfer of disease to monkeys by injection of kidney bound antibodies from Goodpasture patients and the therapeutic effect of treating patients with plasma exchange and immunosuppressive drugs to reduce the amount of circulating antibodies. The Goodpasture epitope is a conformational epitope, which is indicated by the loss of reactivity to autoantibodies when the tertiary protein structure is disrupted by reduction of disulfide bonds. The epitope is also known as a cryptotope, i.e. the epitope is hidden in the native protein structure and is fully exposed first when the protein is partially denatured.

Trying to map an epitope for a specific autoimmune disorder is very difficult, since in most cases autoantibodies against a variety of epitopes on the target structure are formed. For Goodpasture syndrome a limited epitope distribution was indicated when binding of autoantibodies to collagen IV was successfully blocked by one single monoclonal antibody. In our first attempt to map the Goodpasture epitope we used linear synthetic peptides of the α3(IV) NC1 domain to block the binding of autoantibodies to collagen IV. With this method we were unable to map any epitope on the α3(IV) NC1 domain although for one patient an epitope on the α1(IV) NC1 domain was found. In a study by Kalluri et al., using linear peptides, they suggested the C-terminal part of the α3(IV) NC1 domain to comprise the Goodpasture epitope. However this study as well as ours suffered from the disadvantage of using linear peptides to characterize a conformational epitope and the results have not been confirmed.

To avoid the problem with linear peptides and mal-folded recombinants expressed in bacterial systems, new mapping strategies has been initiated by several groups, where recombinant collagen IV is expressed in eukaryotic cell lines. By substitution of amino acid residues in the α3(IV) NC1 against the corresponding ones from the homologous but non-reactive α1(IV) chain, and expressing the constructs in an eukaryotic expression system correctly folded proteins with intact conformational epitopes are produced. All these studies have emphasized the N-terminal part of the α3(IV) NC1 as the principal epitope region. Furthermore, we found that only autoantibodies against the N-terminal third of the α3(IV) NC1 domain are pathologically significant. Following studies have revealed a small region within the N-terminal part of the α3 NC1 as the major target for the circulating antibodies. This epitope has then been recreated by substitution of a few a.a. residues in the non-reactive α1-chain to the corresponding ones from α3.

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FIG. 1. a, a schematic drawing of the cDNAs inserted into the expression vector (pCEP4-BM40-HisEK). This construct result in an expressed protein containing a 6 × His tag followed by a 30-amino acid strech of type X collagen and the NC1 domain from type IV collagen. b, in the basic construct, S1, amino acids in positions P5–P9 are substituted from the α1 ones (upper row) to the corresponding ones of α3(IV) NC1 (lower row). The positions for substitutions in the α1 protein sequence are indicated by arrows and numbers. For example, the R12 construct is substituted in positions P1 and P2 plus P5–P9.

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 Hospital. All patients showed crescentic glomerulonephritis with linear deposits of IgG at direct immunofluorescence. Seven of the patients had in addition overt lung hemorrhage. The mean age was 49 years ranging from 18 to 78 years, 10 males and 10 females. The mean serum creatinin at time of diagnosis was 92.0. Twelve of the patients were maintained on dialysis, four had died and four survived with native functional kidneys after 6 months of follow-up. Sera from five healthy blood donors were used as controls.

Antibodies—The alkaline phosphatase-conjugated antibodies, goat anti-human IgG (Fc part) and goat anti-rabbit IgG, were purchased from Sigma and the rabbit anti-mouse IgG from Dako, Glostrup, Denmark.

The polyclonal rabbit anti-collagen X antibodies were manufactured at the Department of Immunology, University of Copenhagen. The sera of 20 Goodpasture patients with biopsy-proven anti-glomerular basement membrane nephritis were used. The sera were diluted 1/200. The monoclonal antibodies against the type X collagen domain and antibodies against the His tag. Protein concentrations were determined using the BCA (Pierce) method and by measuring the absorbance at 280 nm.

Enzyme-linked Immunosorbent Assay (ELISA)—The microtiter plates (Nunc ImmunoPlate, Roskilde, Denmark) were coated overnight at 4 °C with 0.025 μg/well of purified recombinant protein in coating buffer (50 mM Na2CO3, 0.05% NaN3, pH 9.6). The plates were washed three times with 0.15 M NaCl, 0.05% (v/v) Tween 20 and then incubated for 1-h preincubation at room temperature with 100 μl/well of sera diluted 1:100 in phosphate-buffered saline-bovine serum albumin (1.5 mM KH2PO4, 8 mM NaH2PO4, 0.12 M NaCl, 2.5 mM KCl, 0.05% (v/v) NaN3 containing 0.2% (w/v) bovine serum albumin, pH 7.3). After three new washes the plates were incubated with alkaline phosphatase-conjugated goat anti-human IgG for an additional hour. The amount of bound antibodies was detected by the use of p-nitrophenyl phosphate (Sigma) (1 mg/ml) in substrate buffer (1% diethanolamine, 0.5 mM MgCl2, pH 9.8), as substrate. Color development was measured spectrophotometrically at 405 nm after 1-h incubation at room temperature. All assays were run in duplicate.

Inhibition ELISA—The inhibition ELISA was performed in the same way as described above, with the exception of the preceding overnight preincubation at 4 °C of patient sera with recombinant protein in concentrations of 10 to 0.0001 μg/ml. The plates were coated with recombinant α3-NC1 (0.025 μg/well).

To find the optimal concentration of patient sera for inhibition, two dilution series were made for each of the six patient sera used in the inhibition ELISA. One of the dilution series was preincubated with recombinant α3-NC1 (2 ng/well), and the analogous series was preincubated with an equal volume of phosphate-buffered saline-bovine serum albumin buffer. In this way two "dilution curves" were achieved, where the absorbance for the curve produced from the dilution series affected by inhibition from the α3(IV) NC1 declined earlier and reached background absorbance earlier than the non-inhibited dilution series. The serum dilution that gave the largest difference, in percentages, between the inhibited and non-inhibited dilution series was used in the inhibition ELISA assays for the different recombinants. Antibodies bound to the coat were detected with alkaline phosphatase-conjugated secondary antibodies as described above.
In a previous study we were able to recreate an immunoreactive epitope in the non-reactive $\alpha 1$(IV) NC1 domain by substitution of single amino acids to the corresponding ones of $\alpha 3$ (15). In that study two principle constructs were made, one with five amino acid substitutions in positions known to be critical for epitope recognition (P5–P9), called the S1 construct, and one with four additional substitutions in non-conserved positions in the same region (P1–P4), called S2. It was shown that the five substitution recombinants only reacted weak with the antibodies, whereas the nine-substitution recombinant showed immunoreactivity to all tested sera. In this present study, we have investigated what impact each of these amino acids has on the affinity of autoantibodies from different patients. Thereby further characterizing the immune response in Goodpasture disease to this major epitope. To achieve this, all patients. Thereby further characterizing the immune response in Goodpasture disease to this major epitope. To achieve this, all patients.

**RESULTS**

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**Initial Screening of Reactivity**—Initial tests of the 14 recombinants’ immunoreactivity against all 20 patient sera were performed using ELISA and inhibition ELISA. Of the 14 tested recombinants only five were reactive with the patient sera, one with four additional substitutions in non-conserved positions in the same region (P1–P4), called S2. It was shown that the five substitution recombinants only reacted weak with the antibodies, whereas the nine-substitution recombinant showed immunoreactivity to all tested sera. In this present study, we have investigated what impact each of these amino acids has on the affinity of autoantibodies from different patients. Thereby further characterizing the immune response in Goodpasture disease to this major epitope. To achieve this, all possible combinations of the four additional substitutions were made in a total of 14 new recombinants. The S1 construct was used as template, and then subsequent substitutions were introduced with site-directed mutagenesis using the primers listed in Table I.

**Inhibition ELISA**—In contrast to the direct ELISA, the inhibition ELISA revealed differences in affinity to the different recombinants. The highest inhibitory effect was found using recombinant $\alpha 3$, S2, R12, R123, R124, and R134 and immunoblotted against the anti-type X collagen antibody in A and against one representative Goodpasture serum in B. The arrow indicates the 31-kDa recombinant proteins that appear as double bands, probably due to proteolytic degradation. The control antibody recognizes all recombinants, while the patient serum bound the recombinant $\alpha 3$, S2, R12, R123, and R124. A faint staining is seen against the R134, and no binding was detected against the recombinant $\alpha 1$ or S1.

**DISCUSSION**

Circulating autoantibodies against different parts of the $\alpha 3$(IV) chain, as well as antibodies against other $\alpha$(IV) chains, are detected in serum from patients with Goodpasture disease (6, 19). However, the major epitope region is found to be the N-terminal part of the $\alpha 3$(IV) NC1 (9, 11), and only antibodies against this part of the molecule correlate with disease progression (12, 15). In this study we have further narrowed down and characterized the molecular properties of this epitope recognized by the pathogenic autoantibodies. A recombinant protein comprised of the $\alpha 1$(IV) NC1 domain with seven amino acids substituted to the corresponding ones from the $\alpha 3$ chain was constructed. This recombinant protein, R12, was recognized by the autoantibodies from all patients with Goodpasture disease.

**TABLE I**

Primers used to introduce amino acid substitutions in the collagen IV $\alpha 1$(NC1) domain

| Primer | Sequence 5’ to 3’ |
|--------|------------------|
| 1      | CCAGGGCCCTCCTGCGTCTGGG |
| 2      | CCATTTGGGCCGCTTATGTCCTCTCATACAGAC |
| 3      | AGTCAAAACAGAGTATCCCATGCGTCC |
| 4      | GGGATATGGTCGTTGTTTG |
| 5      | CCGTCTGCCTCAGAGCACGAGTGG |
| 6      | GCCGACGATGGTACGGTCCTCC |
| 7      | CAACAAATAGCTATCCCCATGCGTCC |
| 8      | CGAGGGGATAGCTATTGTTTG |
| 9      | CCATCGTGGCTCCTGGAGGAGGACC |
| 10     | GGGATCTGGTTGTTTGACTATGC |
| 11     | TCTGGGACCGTACCTCTCTTTACAGCGG |
| 12     | CGATGGGATAGCTGGTGTGTTTG |
| 13     | GGAGGGATACCTCTTTACAGCGG |

**SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting**—A 10% NuPAGE gel from Novex (San Diego, CA) was run in a MOPS buffer system according to the supplier’s recommendations. The gels were either silver-stained or transferred to a polyvinylidene difluoride membrane using a semidry blotting procedure (18). The antibodies were diluted as described above, and the membranes were treated as described elsewhere (6).
disease, in both direct ELISA and in inhibition ELISA, to the same degree as recombinant α3. These results support the previous findings where we and others have localized the major epitope to the same region (13–15).

The very limited region recognized by all Goodpasture sera indicates a similar immunization and maturation process in all patients. Interestingly, an overlapping T-cell epitope is found by Phelps and co-workers (20, 21). However, if this process is initiated by a foreign immunogen, e.g., R12, displays an epitope more similar to a hypothetical mimicry structure than the native α3(IV) NC1 does.

Although all samples recognize one very limited area on the α3(IV) NC1, there are some differences in recognition pattern between the different samples. As discussed above the effect of charge changes within the loop have different effect on antibodies from different sera (especially the R124). Furthermore the relative amount of antibodies against the epitope defined by the R12 constructs varies from serum to serum, ranging from 65 to 95%.

We believe that this study of the Goodpasture epitope adds new and important data that will help us to understand the underlying immunological mechanisms in Goodpasture disease in particular, but also for autoimmune diseases in general. By using the R12 recombinant protein in an assay instead of the complete α3(IV) NC1, a more specific diagnostic test could be developed that could distinguish between pathogenic antibodies and harmless autoantibodies.

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