POINT MUTATIONS OF SINGLE AMINO ACIDS ABOLISH ABILITY OF $\alpha_3$ NC1 DOMAIN TO ELICIT EXPERIMENTAL AUTOIMMUNE GLOMERULONEPHRITIS IN RATS

Thomas Hellmark\(^1\)
Lanlin Chen\(^2\)
Sophie Ohlsson\(^1\)
Jörgen Wieslander\(^2\)
Warren Kline Bolton\(^2*\)

\(^1\)Department of Nephrology
Lund University Hospital
S-22185 Lund, Sweden.

\(^2\)Department of Medicine
Division of Nephrology
University of Virginia Health Sciences Center
Post Office Box 800133
Charlottesville, Virginia 22908-0133

*Corresponding author, proofs, and reprint requests:
W. Kline Bolton, M.D.
Post Office Box 800133
University of Virginia Health Sciences Center
Charlottesville, Virginia 22908-0133, USA.
Telephone: 434-924-5125
FAX: 434-924-5848
E-mail: wkb5s@virginia.edu

Running title: Effect of antigen mutation on glomerulonephritis in rats
SUMMARY

We previously showed concordance between Goodpasture syndrome antibody binding and production of experimental glomerulonephritis using human chimeric proteins. We now examine a more limited amino-terminal region of \( \alpha_3(IV) \) non-collagenous domain (NC1) and the impact of single AA mutations of this region on glomerulonephritis induction.

Rats were immunized with collagenase solubilized glomerular basement membrane (csGBM), D3, an \( \alpha_1(IV) \)NC1 chimeric protein with 69 AA of \( \alpha_3(IV) \)NC1 (binds Goodpasture sera), D4, the D3 construct shortened by 4 AA (non-binding), P9 and P10, single AA mutants (non-binding) and S2, \( \alpha_1(IV) \)NC1 with 9 AA of \( \alpha_3(IV) \)NC1 (binding).

All rats immunized with csGBM and S2, and 50% of D3 rats developed glomerulonephritis. csGBM rats had intense GBM-bound IgG deposits, but S2 and D3 rats had minimal deposits. None of D4, P9 or P10 rats developed glomerulonephritis. Lymphocytes from nephritic rats proliferated with csGBM, S2 and D3, but not with D4, P9 or P10.

Discrete segments of \( \alpha_3(IV) \)NC1 within the \( \alpha_1(IV) \)NC1 backbone can induce glomerulonephritis. Single AA mutations within that epitope render the antigen unresponsive to Goodpasture sera and incapable of inducing glomerulonephritis. These studies support concordance of glomerulonephritis inductivity and Goodpasture serum binding. They further define a critical limited AA sequence within \( \alpha_3(IV) \)NC1 of 9 or less AA which confers nephritogenicity to the non-nephritogenic \( \alpha_1(IV) \)NC1 sans \textit{in vivo} antibody binding. This region may be a T-cell epitope responsible for induction of glomerulonephritis in this model in rats and Goodpasture syndrome in man.

Word Count: 236
INTRODUCTION

The epitope responsible for inducing Goodpasture syndrome in man has been localized to the amino terminal third of the \( \alpha_3 \) noncollagenous domain (NC1)\(^1 \) of type IV collagen \[\alpha_3(IV)NC1\] (1-6). Autoimmunization to \( \alpha_3(IV)NC1 \) in man is associated with rapidly progressive glomerulonephritis with deposition of IgG along the glomerular basement membrane (GBM) and tubular basement membrane (7). In some patients with intrinsic pulmonary damage, hemoptysis also occurs (7). The disease is mediated by autoantibodies to type IV collagen as shown by transfer of disease with antibody (8-11). The disease is also T-cell dependent as shown by requirement for intact T-cell immunity, cell transfer in animal models, and immunodominant T-cell epitopes in man (10;12-16).

We have previously shown using chimeric proteins and recombinant constructs of \( \alpha_3(IV)NC1 \) domain that the major nephritogenic epitope for induction of experimental autoimmune glomerulonephritis in rats is also localized to the amino terminal third of \( \alpha_3(IV)NC1 \) domain (17). Point mutations of various AA in this region of \( \alpha_3(IV)NC1 \) are capable of abolishing Goodpasture syndrome antibody binding (18). Whether these same epitopes are also responsible for induction of disease cannot be studied directly in man. We have thus utilized our model of glomerulonephritis to address this question. This model, induced by immunization with native or recombinant GBM antigens, results in severe proliferative glomerulonephritis in rats with crescents, hematuria, proteinuria, pulmonary hemorrhage, and decreased kidney function with progression to chronic kidney failure resulting in death (19). In the present studies, we have extended our investigations of the critical role of the amino terminal third of the NC1 domain in induction of glomerulonephritis. We show that Goodpasture serum binding constructs also induce glomerulonephritis and a T-cell response, while point mutations resulting in abrogation of Goodpasture antibody binding also abrogate the ability of the
constructs to induce glomerulonephritis and elicit T-cell proliferation. Finally, the close approximation of these epitopes within a narrow region in the absence of in vivo bound antibody suggests that this area may contain an epitope responsible for T-cell induced glomerulonephritis.

**EXPERIMENTAL PROCEDURES**

**Antibodies**

Patients with Goodpasture syndrome were used as a source of human autoantibodies. Murine monoclonal antibodies with specificity to the α3(IV)NC1 domain (Mab17), a monoclonal antibody that recognizes a discontinuous conformational epitope on α3(IV)NC1 consisting of AA 17-31 and 127-141 (20), and against 6xHis epitope (anti-His.G) (Qiagen) were used in these experiments. Anti-36 mer antibody is from rats immunized against the terminal 36 AA of α3(IV)NC1 (21). It recognizes both α1 and α3(IV)NC1 domains. Rat sera were obtained from animals immunized with recombinant proteins and collagenase solubilized (cs) GBM. Horseradish peroxidase conjugated antibodies, fluorescein conjugated goat anti-rat IgG and fibrinogen, and antihuman IgG were purchased from ICN/Cappel.

**Electrophoresis and Immunoblotting**

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 12.5% gels under non-reducing conditions. csGBM, 1 mg/ml, and purified recombinant proteins, 0.05 mg/ml, were dissolved in Laemmli buffer and 20 µl loaded in each lane (22). For immunoblotting studies, the proteins separated on SDS-PAGE were transferred to nitrocellulose membranes (BioRad), blocked with 5% dry milk in 0.1% phosphate buffered saline Tween 20 (PBS-T) and washed with PBS-T. The membranes were incubated for 2 hours with primary antibodies diluted in PBS-T. Following this, the membranes were washed
thoroughly with PBS-T, followed by 1-hour incubation with horseradish peroxidase-conjugated secondary antibodies, multiple washing with PBS-T, and identification of proteins by chemiluminescence (Pierce).

**Enzyme-linked immunosorbent assay (ELISA)**

ELISA assays for native and recombinant proteins were performed as previously described in detail (18;19;23;24). All assays were run in duplicate and measured spectrophotometrically at 405 nm.

**Preparation of bovine csGBM**

csGBM immunizing antigen was isolated from homogenized cortical tissue by differential sieving, sonication to obtain disrupted GBM, and consequent digestion with collagenase to form csGBM as previously described (19;23).

**Recombinant human α3(IV)NC1 and chimeric α3/α1(IV)NC1**

Chimeric proteins (Figure 1) were constructed as previously described by substituting various lengths of α3(IV)NC1 chain with the α1(IV)NC1 chain, which is non-nephritogenic (Figure 1A) (1;18). These substitutions consisted variably of different lengths of α3 with predominant α1 designated D3, D4, and single AA mutations from α3 to α1, P9 and P10 (Figure 1B). S2 consists of the α1(IV)NC1 domain with 9 AA from α3(IV)NC1 substituted into the backbone of α1(IV)NC1. csGBM, D3, and S2 all bind Goodpasture sera. D4, P9, and P10 are non-binding with Goodpasture sera (18). Replacement mutations were introduced by site-directed mutagenesis using an overlap extension polymerase chain reaction (18). The constructs were expressed in HEK293 cells. The secreted proteins contained a BM40 signal peptide.
followed by a 6 histidine tag and 30 AA length of type X collagen, and the NC1 domain from type IV collagen. Constructs were tested for protein translation with the correct molecular weight using an in vitro system (Promega, Madison, WI) with \( ^{35}S \) cysteine and T7 RNA polymerase. Cells were cultured in Dulbecco’s modified Eagles medium (DMEM/F-12) with 5% fetal calf serum (Gibco BRL) in the presence of selection reagent G-418 (Gibco BRL). Culture medium was collected and purified by resin column (Invitrogen).

\( \alpha_{3-732} \) is a full-length \( \alpha_3(IV) \)NC1 domain without the collagen X leader protein (25). It was likewise expressed and recombinant protein produced in HEK293 cells. The secreted protein was fused with a myc epitope and 6x histidine tag at the carboxyl-terminus (25). The cells were cultivated, harvested and purified as described above except that zeocin (Invitrogen) was used as the selection reagent. \( \alpha_{3-732} \) induces glomerulonephritis in rats (25). \( \alpha_{3-732} \) purified by high pressure liquid chromatography was used in ELISA and immunoblot experiments in this study.

Characterization of the proteins demonstrated that Mab 17 was blot positive with protein containing AA 17-31 and 127-141, which are required for its binding (5;6) (csGBM, \( \alpha_{3-732} \)) but negative for constructs lacking the second site (S2, D3, D4, P9, P10) (Figure 2). All constructs blotted positive with anti-36 mer, at the expected molecular size (Figure 2). Previous studies demonstrated binding of Goodpasture sera to S2 and D3, but not to D4, P9, or P10 (18).

**Experimental Animals and immunization**

Hypersusceptible normal male WKY rats, weighing 200 g, were obtained from Charles River Laboratories (Wilmington, MA, USA). The animals were kept in standard conditions in the Department of Comparative Medicine for acclimatization prior to initiation of the experimental studies. The immunizing antigens were suspended in 0.1M acetic acid, emulsified
with an equal amount of complete Freunds adjuvant (H₃7Ra) (Sigma) and given as a single injection in the left hind footpad and subcutaneously. Each animal received 100 µg of protein. There were six to seven rats in each experimental group.

Serum biochemistries, urine analysis and total urinary protein

Animals were placed in metabolic cages and 24-hour urine samples collected weekly following immunization for a total of 7 weeks. Urinary protein was determined using 3% sulfosalicylic acid with bovine serum albumin (BSA) as a standard (19). The upper limits of normal per 24 hours are 10 mg. Urine was also examined for hematuria (0-3+) using reagent strips (Multistix 10 SG, Bayer Corporation). Serum creatinine and urea nitrogen were measured in blood obtained from the tail vein from each rat every 2 weeks following immunization using diagnostic kits from Sigma for creatinine (Procedure No.555) and urea nitrogen (Procedure No. 640).

Immunofluorescence studies and histological examination

Kidney tissue obtained at death or at sacrifice at eight weeks after immunization was used for histologic studies. Tissue was snap-frozen in isopentane (2-methylbutane, Fisher) on dry ice and stained for rat IgG and fibrinogen as previously described (26). The intensity of deposits was semiquantitatively graded in a masked fashion from 0 to 4+ (19;23;27). For light microscopy studies, kidney tissue was fixed in 10% buffered formalin, dehydrated in alcohol, and embedded in paraffin. Hematoxylin-eosin stained sections were examined in a masked fashion using a 4-point scale (26).
Lymphocyte-Proliferation Assay

Lymphocytes isolated from spleens of rats with glomerulonephritis were stimulated in vitro with different antigens and cultured in 96-well flat-bottom plates as previously described (21;26). Three days later, the cells were pulsed with tritiated thymidine, harvested, and counted in a liquid scintillation counter. All assays were performed in triplicate. Data are expressed as the stimulation index, the ratio of stimulated to medium counts per minute (CPM). We considered stimulation indices of 2.0 or greater as significant (26).

Statistical Analyses

Data are expressed as mean ± SE. Statistical differences between groups were evaluated by the Student’s t-test and analysis of variance (23).

RESULTS

Induction of Glomerulonephritis by csGBM and Goodpasture Serum Binding Chimeric Protein Constructs

Animals immunized with csGBM and S2 construct all developed hematuria and half of the animals immunized with D3 developed hematuria. csGBM and S2 immunized rats developed proteinuria in a typical fashion with onset by 3 weeks (Figure 3). Approximately half of the animals immunized with D3 also developed proteinuria albeit of lesser degree than S2 and csGBM immunized animals. Elevations of blood urea nitrogen and serum creatinine were observed in csGBM and S2 immunized animals (Figure 4A,B) but not D3 immunized rats.

By light microscopy, all animals immunized with csGBM and S2 had typical florid glomerulonephritis (Figure 5). This was characterized by intense tubulointerstitial infiltration with mononuclear cells, tuftal proliferation with fibrosis, scarring, cellular crescents with giant
cells and tubular atrophy. Half of the D3 immunized animals developed overt glomerulonephritis as well, with the intensity of histologic findings being somewhat less than that observed with S2 and csGBM.

By immunofluorescence (Figure 6), intense linear deposits of IgG typical of glomerulonephritis were observed on the basement membrane in csGBM immunized animals. In contrast to csGBM recipient rats, animals immunized with S2 and D3 had minimal deposits of IgG along the basement membrane despite glomerulonephritis. Five of 7 S2 rats were completely negative while two had slight deposits. One D3 immunized rat had 1-2+ GBM deposits of IgG, two had trace deposits, and three were negative. Typical fibrinogen deposits were present in csGBM, D3, and S2 immunized animals. The fibrinogen deposits were present in all csGBM and S2 rats, and were more intense in csGBM and S2 immunized animals than D3 rats. Five of six D3 immunized animals had fibrinogen deposits and three of these rats had glomerulonephritis by light microscopy.

**Influence of Mutations Abolishing Goodpasture Serum Binding on Glomerulonephritis Induction**

Rats immunized with D4, P9, and P10 did not show any evidence of hematuria at any time. No proteinuria or biochemical abnormalities were observed in these animals, there were no histologic abnormalities, and no deposition of antibody or fibrinogen in glomeruli.

No deposits, biochemical abnormalities, or histologic abnormalities were observed in negative control Freunds adjuvant immunized rats.

**Lymphocyte Proliferation**
Cells from rats with glomerulonephritis responded strongly to antigens with Goodpasture serum binding capacity (Table 1). On the other hand, constructs which failed to bind Goodpasture sera in vitro (18) also failed to elicit cellular proliferation.

**Antibody Reactivity**

**ELISA**

All animals immunized with chimeric proteins containing collagen X leader protein produced a vigorous antibody response in ELISA against the immunogen, collagen X, α3-732, and other chimeric constructs (Figure 7). They also demonstrated a robust response to NC1 domains from bovine and human GBM, and lesser responses to rat NC1 and csGBM. At a common dilution of 1:1000 vs bovine NC1, the week six sera optical densities were respectively 0.69 for rats immunized with csGBM, 0.49 for P9, 0.25 for P10, 0.46 for D3, 0.41 for S2, and 0.42 for D4. At a dilution of 1:4000, all sera had optical densities comparable to normal serum.

**Indirect Immunofluorescence**

By indirect fluorescence of kidney sections, Table 1, staining of human GBM was present with sera from csGBM immunized rats. In addition, P9 and P10 immunized animals without glomerulonephritis also had circulating antibody to human glomeruli in tissue sections. S2 and D3 immunized rats sera had much less activity against GBM sections in vitro. There was no circulating antibody activity in D4 and Freund’s adjuvant immunized rats by indirect immunofluorescence on human kidney sections, despite antibody by ELISA. The fluorescence pattern was both GBM and mesangial for sera from D3 and P9. None of the sera from any of the rats bound to rat GBM in sections of rat kidney by indirect fluorescence.
DISCUSSION

We have shown that the amino terminal domain of \( \alpha_2(IV)NC1 \) contains the immunodominant region for binding antibodies from patients with Goodpasture syndrome (1). This does not prove that the epitope is also capable of inducing disease, nor are these studies possible in man. The purpose of the present study was to determine whether the same antibody binding epitope was responsible for induction of disease in our model. Indeed, constructs demonstrating in vitro antibody binding also induced glomerulonephritis. Somewhat surprising to us was the ability of single point mutations in P9 and P10 constructs to totally abrogate the ability of the chimeric proteins to induce glomerulonephritis. While this was consistent with in vitro Goodpasture antibody binding (18), we had anticipated that redundancy in the immune system would nonetheless allow these constructs to induce glomerulonephritis. Animals immunized with the P9 and P10 constructs did develop antibody to human GBM in vitro, while D4 immunized animals developed no antibody to human GBM in tissue sections but did by ELISA. This illustrates the key importance of the four AA (TAIP) contained in the D3 construct for both disease induction and Goodpasture antibody binding.

To examine the critical AA further, S2 was utilized to immunize rats. Previous studies with chimeric protein S1 containing five of the critical AA on the \( \alpha_1(IV)NC1 \) backbone demonstrated non-binding of Goodpasture antibodies (18). Four additional AA substitutions for a total of nine AA from \( \alpha_3(IV)NC1 \) was sufficient to restore full antibody binding activity to the construct, S2 (18). Similarly, these same nine critical AA on the \( \alpha_1 \) NC1 backbone restored nephritogenicity to the chimeric protein construct such that disease severity in S2 immunized animals was the same as animals immunized with csGBM. However, there was one significant difference. Rats immunized with csGBM had deposits of rat IgG along the GBM in vivo and circulating antibody which fixed avidly to human GBM in vitro. S2 immunized rats had minimal
deposits of IgG in vivo and serum from these animals reacted less intensely by indirect fluorescence with human GBM.

The D3 construct containing the same 9 critical AA as S2 and native csGBM induced glomerulonephritis as well, with minimal deposits of IgG on the GBM, like S2, but lesser amounts of fibrinogen and less severe glomerulonephritis. We expected comparable glomerulonephritis production with D3. While we do not know the reason for lesser nephritis with D3, we believe that flanking regions both proximal and distal to the critical nephritogenic epitope influence disease expression (17). These flanking regions would differ between D3 and both csGBM and S2.

Induction of disease with the nine AA substitutions, eight of which are contained within a region spanning fifteen AA, suggests the possibility that this region may contain a T-cell epitope (28;29). Lymphocytes from glomerulonephritic rats proliferated to constructs containing these AA, but not to the other constructs. T-cells have been shown to be necessary and sufficient for production of disease as demonstrated by transfer studies with mononuclear cells, and the requirement for an intact T-cell immune system for induction of glomerulonephritis and antibody formation (10;12-14;30-32). T-cells also transfer antibody production so study of the individual antibody and cellular mediated systems has been difficult (10). Induction of glomerulonephritis without apparent GBM bound antibody strongly implicates a cell mediated mechanism (23;30;33;34).

While a number of in vitro Goodpasture T-cell epitopes have been described (15;16), the pathogenicity of those epitopes is not known. In the present studies we have shown that nine essential AA are sufficient to induce Goodpasture syndrome in this animal model, and that eight of these nine critical AA comprise a sequence consistent with the size of a T-cell epitope. Since the nine AA are superimposed on the backbone of the α1(IV)NC1 domain which does not cause
glomerulonephritis (35),(17;36) they must be assumed to be critical for the pathogenic capacity of the $\alpha_3$ NC1 chain. The critical nature of this epitope is demonstrated by single AA point mutations in two of them completely abrogating the ability to bind antibody, elicit lymphocyte proliferation or induce glomerulonephritis. They are thus critical not only in the conformation of the protein required for antibody binding, but in the epitope required for induction of disease. Single AA mutations of immunogen in a model of oophoritis can abrogate disease by interference with the responsible T-cell epitope (37). The observations in the present studies suggest a similar fine specificity of antigen in this model.

Goodpasture syndrome has classically been considered an antibody mediated process. Discrepancies between circulating and GBM bound antibody and clinical presentation have received scant attention. Thus, the observations on in vivo and in vitro antibody activity are of interest. Because of homology between $\alpha_3$(IV)NC1 and $\alpha_1$(IV)NC1, antibody cross-reactivity in ELISA might be expected with both recombinant and native proteins (38). In addition, the presence of collagen X in most constructs, and 6-histidine in all, would contribute to ELISA positivity. The findings by direct and indirect immunofluorescence on kidney sections help to interpret the observations of antibody reactivity using different substrates. csGBM positive control rats had strong IgG deposits in vivo, S2 and D3 essentially none. While most rats developed circulating antibody to human GBM, none had circulating antibodies which bound in vitro to rat kidney sections. The findings in the present studies are consistent with previous reports. Antibody titers peak at 4-8 weeks and then plateau or decline (39-41). The amount of IgG bound to rat GBM in vivo is very variable, ranging from 0 to 4+, and correlates poorly with disease, as some animals may have minimal or no deposits, yet florid disease (21;30;33;36;39;41-45). Antibody deposits may diminish with time as glomerular sclerosis occurs and/or rats die, resulting in lower average fluorescence scores (39;42;43;46). It is
unlikely that glomerular bound antibody was present but undetected in our studies, as we have previously shown we can detect <6 fg/gomerulus and <0.01 µg/gm tissue of IgG (23). This is far less than the amount of homologous or heterologous GBM bound antibody required to induce glomerulonephritis (47). Finally, the histologic score by light microscopy reflects the aggregate of damage, whether by antibody, cell mediated immunity, or combined mechanisms. Circulating antibodies in rats may have reactivity with GBM components by ELISA and immunoblot. However, they are frequently weak or absent by indirect immunofluorescence on rat kidney sections and are insufficient, when present, to transfer disease, even though strongly positive vs other species kidney sections (8;21;33;45). Further, linear GBM deposits in animal models and man may occur, but with minimal or no disease (10;39;42;44;48).

These various observations emphasize there are multiple epitopes capable of inducing antibody formation, some species of which bind to native protein (kidney sections), others to altered protein (ELISA, immunoblots), and they may or may not induce disease. The autoreactive antibodies represent only a portion of total antibodies, and many circulating antibodies react with epitopes unique to the immunogen but irrelevant to the disease process. In addition, immunization with antigen may induce antibodies, but with disease actually caused by cellular immunity (30;33;49). Finally, antibody negative glomerulonephritis can be produced both with peptides and cell transfer (10;12;30). It is thus essential to establish the link between the epitope and the disease, not between the epitope and epiphenomenon, i.e. antibody.

The findings of a discrete region of the α3(IV)NC1 domain consisting of these few AA with consequent disease but minimal antibody activity raises the possibility that two or more epitopes may be involved in induction of the disease. One, a B cell epitope, may well consist of one or more portions of the α3(IV)NC1 domain as suggested by Goodpasture serum binding to two discrete segments of the α3 domain in the amino terminal and middle thirds of α3(IV)NC1
[AA 17-31 and/or 127-141 (1;5;6)]. Another, T-cell epitope, may be the same B cell epitope, or other region. Identification of both the T- and B-cell epitopes is critical for understanding the interaction between T-cell and antibody mediated immunity both in this experimental animal model and in man, and for development of therapeutic constructs which could be used for patients with Goodpasture syndrome.
ACKNOWLEDGMENTS

This work was supported by USPHS Grant DK 55801 from the NIDDK, K2001-71X-09487-11C from the Swedish Research Council, the Tegger Foundation and the Swedish Society for Medical Research (SSMF). This work was presented in part at the 33rd Annual meeting of the American Society of Nephrology 2000. We thank the Biomolecular Research Facility at University of Virginia for their expert support on protein sequencing, and Dr. Peter Lobo for collaboration with the cell proliferation assays. We are grateful to Ms. Chun Gao for her excellent technical assistance. We thank Patsy Craig for secretarial assistance and Lena Gunnarsson for technical assistance.
REFERENCES


FOOTNOTES

1 – The abbreviations used are:

BSA - bovine serum albumin

CPM - counts per minute

csGBM - collagenase solubilized glomerular basement membrane

D3 - Goodpasture positive serum binding construct

D4 - Goodpasture negative serum binding construct

ELISA - enzyme-linked immunosorbent assay

GBM - glomerular basement membrane

NC1 - non-collagenous domain

PBS - Phosphate buffered saline

PBS-T - PBS-Tween

P9 - Goodpasture negative serum binding construct

P10 - Goodpasture negative serum binding construct

SDS-PAGE - Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

S2 - Goodpasture positive serum binding construct
FIGURE LEGENDS

Figure 1. Cartoon demonstrating the cloning strategy for constructing human α3/α1 chimeric proteins and point mutations. (1;24) A) the chimeric construct D1 consists of a signal peptide, 6 histidine tag, type X collagen triple helix region of 30 AA, 98 amino terminal AA of α3(IV)NC1 (from A to G) followed by the homologous region of α1(IV)NC1 domain until the end of the construct. The expanded AA sequence shown in Figure 1A provides specific AA in the region A to G, indicates the NC1 start site, and the mutations made in α3(IV)NC1 to α1(IV)NC1, and in α1(IV)NC1 to α3(IV)NC1(S2).

Figure 1B schematically illustrates the constructs in Figure 1A. Thus, D3 consists of α1(IV)NC1 chain from A to C, α3(IV)NC1 from C to G followed by α1(IV)NC1. D4 consists of α1(IV)NC1 from A to D, α3(IV)NC1 from D to G and then α1(IV)NC1. P9 and P10 are point mutations from α3(IV)NC1 to α1(IV)NC1 as indicated. S2 consists entirely of α1(IV)NC1 with nine AA from α3(IV)NC1 and binds Goodpasture sera. S1 consists entirely of α1(IV)NC1 with five AA from α3(IV)NC1, and does not bind Goodpasture sera.

Figure 2. Immunoblotting of different constructs with Mab 17 and anti-36 mer. Lanes were loaded with 20 μl antigen (1 mg/ml csGBM, 0.05 mg/ml chimeric protein) in Laemmli buffer. Proteins were separated by SDS-PAGE under non-reducing conditions, transferred to nitrocellulose membranes, and blotted with Mab 17 or anti-36 mer. Mab 17 recognizes constructs containing both AA 17-31 and 127-141 of α3(IV)NC1, and anti-36 mer recognizes the carboxy termini of α1 and α3(IV)NC1. Constructs with only one site are negative with Mab 17, whereas all are positive with anti-36 mer. P9, α3-732, and csGBM formed dimers, D. All constructs formed monomers, M. Some proteins underwent degradation during isolation, and large molecular weight aggregates were observed with α3-732 and csGBM.

Figure 3. Proteinuria in immunized rats. The total urinary protein of each animal and each immunization group (csGBM •, S2 ○, D3 □, D4 ▲, P9 ★, P10 ○, CFA +) was determined weekly. Results presented as the mean proteinuria of each group. Normal proteinuria for this age of rats is < 10 mg/24 h.
Figure 4. Level of serum urea nitrogen (A) and creatinine (B) in rats immunized with recombinant proteins and csGBM, week eight.

Figure 5. Histologic scores on hematoxylin-eosin sections. One csGBM rat died and tissue could not be assessed.

Figure 6. Immunofluorescence deposits of IgG (□) and fibrinogen (□), mean ± SEM.

Figure 7. ELISA demonstrating reactivity of serum from various construct immunized rats toward recombinant proteins and native NC1 domains. Serum antibody binds specifically to recombinant and native NC1 domains purified from rat (rNC1), bovine (bNC1), and human (hNC1) GBM. Binding to collagen X and constructs containing collagen X and/or 6-histidine is expected of chimeric protein immunized rats. (Serum from rats immunized with: csGBM □, S2 □ D3 □, D4 □, P9 □, P10 □□)
TABLE 1. *In vivo* and *In vitro* Immunoreactant Correlations

<table>
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<tr>
<th>Antigen</th>
<th>In vivo fluorescence&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Indirect fluorescence for IgG on kidney sections</th>
<th>ELISA Goodpasture serum binding (1:18)</th>
<th>Stimulation&lt;sup&gt;b,c&lt;/sup&gt; index</th>
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<sup>a</sup> Fluorescence intensity graded on a 0-4+ (+++++) scale

<sup>b</sup> Stimulation index calculated as \[
\frac{\text{antigen stimulated CPM} - \text{control CPM}}{\text{control CPM}}
\]

<sup>c</sup> Antigens for lymphocyte proliferation assay: csGBM 500 µg/ml; all chimeric proteins 100 µM; 200 µl/well.

<sup>d</sup> +/-mesangium – deposits on both the GBM and mesangium

<sup>e</sup> N/D = not done
Fig. 1
Fig. 2
Fig 3
Fig 4a

Diagram showing Urea Nitrogen levels across different immunization groups (CFA, csGBM, S2, D3, D4, P9, P10) for Week 4, Week 6, and Week 8.
Fig 4b
Fig 6
Fig 7
Point mutations of single amino acids abolish ability of α3 NC1 domain to elicit experimental autoimmune glomerulonephritis in rats
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J. Biol. Chem. published online September 11, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M211951200

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