Point Mutations of Single Amino Acids Abolish Ability of α3 NC1 Domain to Elicit Experimental Autoimmune Glomerulonephritis in Rats

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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 α3(IV) non-collagenous domain (NC1) and the impact of single amino acid (AA) mutations of this region on glomerulonephritis induction. Rats were immunized with collagenase-solubilized glomerular basement membrane (csGBM), D3, an α3(IV)NC1 chimeric protein with 69 AA of α3(IV)NC1 (binds Goodpasture sera), D4, the D3 construct shortened by 4 AA (non-binding), P9, P10, single AA mutants (non-binding), and S2, α3(IV)NC1 with 9 AA of α3(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 the 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 α3(IV)NC1 within the α3(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 the concordance of glomerulonephritis inductivity and Goodpasture serum binding. Further, they define a critical limited AA sequence within α3(IV)NC1 of nine or fewer AA, which confers nephritogenicity to the non-nephritogenic α3(IV)NC1 without 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.

The epitope responsible for inducing Goodpasture syndrome in man has been localized to the amino-terminal third of the α3 noncollagenous domain (NC1) of type IV collagen (α3(IV)NC1) (1–6). Autoimmunization to α3(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 the transfer of disease with antibody (8–11). The disease is also T-cell dependent, as shown by the requirement for intact T-cell immunity, cell transfer in animal models, and immunodominant T-cell epitopes in man (10, 12–16).

By using chimeric proteins and recombinant constructs of α3(IV)NC1 domain, we have previously shown that the major nephritogenic epitope for induction of experimental autoimmune glomerulonephritis in rats is also localized to the amino-terminal third of the α3(IV)NC1 domain (17). Point mutations of various AA in this region of α3(IV)NC1 are capable of abolishing Goodpasture syndrome antibody binding (18). Whether these same epitopes are also responsible for the 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 the induction of glomerulonephritis. We show that Goodpasture serum binding constructs also induce glomerulonephritis and a T-cell response, whereas 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

α3(IV)NC1, α3 NC1 of type IV collagen; GBM, glomerular basement membrane; AA, amino acid; csGBM, collagenase-solubilized GBM; PBS, phosphate buffered saline; PBS-T, PBS-Tween; ELISA, enzyme-linked immunosorbent assay; D3, Goodpasture positive serum binding construct; D4, Goodpasture negative serum binding construct; P9, -10, Goodpasture negative serum binding construct; S2, Goodpasture positive serum binding construct.

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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 consisting of AA 17–31 and 127–141 provides specific AA in the region A–G and indicates the NC1 start site and the mutations made in α3(IV)NC1 to α3(IV)NC1 and in α3(IV)NC1 to α3(IV)NC1(S2).

Electrophoresis and Immunoblotting—SDS-PAGE was performed in 12.5% gels under non-reducing conditions. 1 mg/ml csGBM and 0.05 mg/ml purified recombinant proteins were dissolved in Laemmli buffer, and 20 μl were loaded in each lane (22). For immunoblotting studies, the proteins separated on SDS-PAGE were transferred to nitrocellulose membranes (Bio-Rad), 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 h with primary antibodies diluted in PBS-T, followed by 1-h incubation with horseradish peroxidase-conjugated secondary antibodies, multiple washings 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 described previously 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 described previously (19, 23).

Recombinant Human α3, IV, and Chimeric α3, α4(IV)NC1—Chimeric proteins (Fig. 1) were constructed as described previously by substituting various lengths of α3(IV)NC1 chain with the α3(IV)NC1 chain, which is non-nephritogenic (Fig. 1A) (1, 18). These substitutions consisted variably of different lengths of α3, with the predominant α3, designated D3 and D4, and single AA mutations from α3 to α3, P9 and P10 (Fig. 1B). S2 consists of the α3(IV)NC1 domain with nine AA from α3(IV)NC1 substituted into the backbone of α3(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-His tag, a 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 [35S]cysteine and T7 RNA polymerase. Cells were cultured in Dulbecco’s modified Eagles medium (DMEM/F-12) with 5% fetal calf serum (Invitrogen) in the presence of selection reagent G-418 (Invitrogen). Culture medium was collected and purified by resin column (Invitrogen).

α3,732 is a full-length α3(IV)NC1 domain without the collagen X leader protein (25). It was likewise expressed, and recombinant protein was produced in HEK293 cells. The secreted protein was fused with a myc epitope and a 6xHis 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. α3,732 induces glomerulonephritis in rats (25). α3,732 purified by high-pressure liquid chromatography was used in the ELISA and immunoblot experiments in this study.
whereas all are positive with anti-36 mer. P9, /H9251

posits was semiquantitatively graded in a masked fashion from 0 to 4
/H11001

IgG and fibrinogen as described previously (26). The intensity of de-
/H2851

in isopentane (2-methylbutane, Fisher) on dry ice and stained for rat

immunization was used for histologic studies. Tissue was snap-frozen

from each experimental group. Each animal received 100

and given as a single subcutaneous injection in the left hind footpad.

Characterization of the proteins demonstrated that mAb17 was blot

positive with protein containing AA 17–31 and 127–141 of

3(IV)NC1. Constructs with only one site are negative with mAb17,

and anti-36 mer recognizes the carboxyl termini of

3(IV)NC1, and anti-36 mer recognizes the carboxyl termini of

a3(IV)NC1 and csGBM.

Fig. 2. Immunoblotting of different constructs with mAb17 and anti-36 mer. Lanes were loaded with 20 µl of 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 mAb17 or anti-36 mer.

mAb17 recognizes constructs containing both AA 17–31 and 127–141 of

β3(IV)NC1, and anti-36 mer recognizes the carboxyl termini of α3 and

α3(IV)NC1. Constructs with only one site are negative with mAb17,

whereas all are positive with anti-36 mer. P9, α3,732, and csGBM

forms 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.

Experimental Animals and Immunization—Hypersusceptible nor-

mal male Wistar-Kyoto rats, weighing 200 g, were obtained from

Charles River Laboratories (Wilmington, MA). 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 0.1 M acetic acid, emulsified

with an equal amount of complete Freund’s adjuvant (H37Ra, Sigma),

and given as a single subcutaneous injection in the left hind footpad.

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-h urine samples were

collected weekly after immunization for a total of 7 weeks. Urinary

protein was determined by using 3% sulfosalicylic acid with bovine

serum albumin as a standard (19). The upper limits of normal per 24 h

are 10 mg. Urine was also examined for hematuria (0–3+) using rea-
gent strips (Multistix 10 SG, Bayer Corp.). Serum creatinine and urea

nitrogen were measured in blood obtained from the tail vein from each

rat every 2 weeks after 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 time of euthanasia at 8 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 described previously (26). The intensity of de-

posits 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 described previ-

ously (21, 26). 3 days later, the cells were pulsed with tritiated thymi-
dine, 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. We con-

sidered stimulation indices of 2.0 or greater as significant (26).

Statistical Analyses—Data are expressed as mean ± S.E. 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 immu-
nized with csGBM and S2 construct all developed hematuria, and half of the animals immunized with D3 developed hematuria.

Animals immunized with csGBM and 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 (Fig. 4, A and B) but not in D3-immunized rats.

By light microscopy, all animals immunized with csGBM and S2 had typical florid glomerulonephritis (Fig. 5), which was characterized by intense tubulointerstitial infiltration with mononuclear cells, tuft 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 some-

what less than that observed with S2 and csGBM.

By immunofluorescence (Fig. 6), intense linear deposits of IgG typical of glomerulonephritis were observed on the base-

ment membrane in csGBM-immunized animals. In contrast to
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Fig. 4. Level of serum urea nitrogen (A) and creatinine (B) in rats immunized with recombinant proteins and csGBM, week 8.

csGBM recipient rats, animals immunized with S2 and D3 had minimal deposits of IgG along the basement membrane despite glomerulonephritis. Five of seven S2 rats were completely negative, whereas 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 there was no deposition of antibody or fibrinogen in glomeruli.

No deposits, biochemical abnormalities, or histologic abnormalities were observed in negative control Freund’s adjuvant-immunized rats.

**Lymphocyte Proliferation**—Cells from rats with glomerulonephritis responded strongly to antigens with Goodpasture serum binding capacity (Table I). On the other hand, constructs that 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, αs1(IV), and other chimeric constructs (Fig. 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 versus 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 with normal serum.

**Indirect Immunofluorescence**—By indirect fluorescence of kidney sections, Table I, 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 rat 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 αs1(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 the 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 the 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. Although 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, whereas D4-immunized animals developed no antibody to human GBM in tissue sections, but they 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 αs1(IV)NC1 backbone demonstrated non-binding of Goodpasture antibodies (18). Four additional AA substitutions (for a total of nine AA from αs1(IV)NC1) was sufficient to restore full antibody binding activity to the S2 construct (18). Similarly, these same nine critical AA on the αs1 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 nine 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. Although 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.
are contained within a region spanning 15 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).

Although 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 critical AA compose a sequence consistent with the size of a T-cell epitope. Because the nine AA are superimposed on the backbone of the $\alpha_3$(IV)NC1 domain that does not cause glomerulonephritis (17, 35, 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

**TABLE I**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>In vivo fluorescence$^a$</th>
<th>Indirect fluorescence for IgG on kidney sections</th>
<th>ELISA Goodpasture serum binding (1, 18)</th>
<th>Stimulation$^{b,c}$ index</th>
</tr>
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<tbody>
<tr>
<td>csGBM</td>
<td>++</td>
<td>+++</td>
<td>–</td>
<td>20/20</td>
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<td>S2</td>
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<td>Freund’s adjuvant</td>
<td>0</td>
<td>0/+++</td>
<td>–</td>
<td>N/D</td>
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</table>

$^a$ Fluorescence intensity graded on a 0–4+ (++++) scale.

$^b$ Stimulation index calculated as $\frac{\text{antigen stimulated CPM} - \text{control CPM}}{\text{control CPM}}$.

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

$^d$ +/mesangium = deposits on both the GBM and mesangium.

$^e$ N/D, not done.
two of them, completely abrogating the ability to bind antibody, elicit lymphocyte proliferation, or induce glomerulonephritis. They are thus critical not only in the confirmation of the protein required for antibody binding but in the epitope required for induction of the disease. Single AA mutations of immunogen in a model of ophoritis 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.

Classically, Goodpasture syndrome has 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 $\alpha_4$ (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-His in all, would contribute to a positive ELISA. 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, and S2 and D3 had essentially none. Although most rats developed circulating antibody to human GBM, none had circulating antibodies that 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 greatly variable, ranging from 0 to 4+, and correlates poorly with disease, as some animals may have minimal or no deposits yet have 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/glomerulus and <0.01 $\mu$g/gm tissue of IgG (23). This amount is far less than the amount of homologous or heterologous GBM-bound antibody required to induce glomerulonephritis (47). Finally, the histologic score by light microcopy 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 versus 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 that there are multiple epitopes capable of inducing antibody formation, some species of which bind to native protein (kidney sections), others to altered protein (ELISA, immunoblot), and they may or may not induce disease. The autoreactive antibodies represent only a portion of the 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, 44, 50). It is thus essential to establish the link between the epitope and the disease and not between the epitope and epiphenomenon, i.e. antibody.

The findings of a discrete region of the $\alpha_3$ (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 the induction of the disease. One epitope, a B-cell epitope, may well consist of one or more portions of the $\alpha_3$ (IV)NC1 domain, as suggested by Goodpasture serum binding to two discrete segments of the $\alpha_3$ domain in the amino-terminal and the middle third of $\alpha_3$ (IV)NC1 (AA 17–31 and/or 127–141; Refs. 1, 5, and 6). Another, a T-cell epitope, may be the same B cell epitope or another 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 the development of therapeutic constructs which could be used for patients with Goodpasture syndrome.
Acknowledgments—We thank the Biomolecular Research Facility at the University of Virginia for their expert support on protein sequencing and Dr. Peter Lobo for collaboration with the cell proliferation assays. We thank Ms. Chun Gao for her excellent technical assistance, Patsy Craig for secretarial assistance, and Lena Gunnarsson for technical assistance.

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doi: 10.1074/jbc.M211951200 originally published online September 11, 2003

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