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Induction of Heymann Nephritis with a gp330/ Megalin Fusion Protein

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There is considerable evidence that glomerular deposits in Heymann nephritis, a rat model of membranous nephritis, result from shedding of immune complexes formed on podocytes and that the principal antigen is part of the extracellular domain of a cell surface glycoprotein receptor called gp330 or megalin. It has also been reported that the immunogen that induces Heymann nephritis is a complex formed between gp330 and the receptor-associated protein RAP. The recent elucidation of the primary structure of gp330 makes it possible to investigate the ability of defined portions of gp330, devoid of RAP, to induce Heymann nephritis. In the present study we show that a gp330-glutathione-S-transferase fusion protein, containing 137 amino acid residues (1114 to 1250) of the ectodomain, induces active Heymann nephritis and that heterologous antibodies against this fusion protein produce passive Heymann nephritis. By immunofluorescence, typical glomerular immunoglobulin deposits were found, but complement components were lacking and the rats did not develop proteinuria. In the active model, we obtained evidence indicating that the deposits contained portions of the ectodomain of gp330, including regions other than those of the fusion protein. Thus, the deposits were stained by polyclonal antibodies to gp330 and to the gp330 fusion protein, as well as by two monoclonal antibodies reactive with portions of the ectodomain of gp330, only one of which reacted with the fusion protein* in vitro. *Antibodies against the cytoplasmic domain of gp330 did not stain. Furthermore, we found that RAP was able to bind to gp330 in the glomerular deposits but not to the gp330 fusion protein* in vitro. *The results show that the region of gp330 spanning amino acid residues 1114 to 1250 contains peptides capable of inducing pathogenic antibodies of Heymann nephritis without a contributory role of RAP. (Am J Pathol 1996, 148:1613–1623)

Heymann nephritis[1] is an experimental model of membranous glomerulonephritis, which can be induced in rats by immunization with certain renal membrane preparations (active, autoimmune Heymann nephritis) or by administration to normal rats of heterologous antibodies against renal antigens (passive Heymann nephritis). The glomerular lesions are characterized by subepithelial immune deposits, which are thought to result mainly from shedding of antigen-antibody complexes formed on podocytes.

There is compelling evidence that the major pathogenic antigen in Heymann nephritis is a large cell surface glycoprotein receptor, usually called gp330 (because its mass was first estimated to be approximately 330 kd,[2] although other estimates of its mass were higher[3]). However, the glomerular lesions induced by immunization with preparations containing mainly gp330 are generally milder than those induced with crude renal membrane preparations,[4] commonly an extract called Fx1A.[5] In particular, abnormal proteinuria usually does not develop in gp330-induced disease, and the glomerular deposits lack complement components, whereas these are characteristic (but not invariable)[6] features of Fx1A-induced Heymann nephritis. These observations have led to a search for additional pathogenic antigens in active Heymann nephritis. However, despite reports describing pathogenic renal antigens smaller than gp330, these have generally not been well characterized and most probably represent breakdown products of gp330.[7]

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Findings concerning the role of gp330 in passive Heymann nephritis have paralleled to some extent those in the active model. Thus, heterologous antibodies against purified gp330 produce glomerular deposits of IgG, but the deposits do not contain complement components and the rats do not develop proteinuria.[8] In contrast, heterologous antibodies prepared against Fx1A, which recognize several proteins in addition to gp330,[9] can induce immune deposits with complement components, often associated with proteinuria.[9,10] Recently, Susani et al[8] have reported that a combination of antibodies to gp330 and to a renal glycolipid are required to produce complement activation and proteinuria in passive Heymann nephritis.

Lately, attention has focused on the role in Heymann nephritis of a well defined 39- to 44-kd protein, called the receptor-associated protein (RAP). This protein binds avidly to gp330, and virtually all purified preparations of gp330, obtained by lectin or immunoaffinity chromatography, contain some RAP,[11–14] a fact that must be considered in experiments designed to induce active Heymann nephritis by immunization with gp330 or passive disease by antibodies to gp330.

It has been shown that RAP combines with gp330 shortly after biosynthesis and that most RAP remains within the endoplasmic reticulum.[15–17] However, there is evidence that small amounts may reach the cell surface.[18,19] Farquhar and associates[11,18] have proposed that RAP combined with gp330 on the podocyte surface constitutes the Heymann nephritis antigen complex. In support of this hypothesis, they have reported that immunization of rats with a RAP fusion protein or passive administration of antibodies to this protein can induce glomerular deposits characteristic of Heymann nephritis.[20,21] Furthermore, they have identified a region of RAP that contains nephritogenic epitopes.[21] However, antibodies to RAP have not been shown to play a role in active Heymann nephritis induced with Fx1A.[14] Furthermore, gp330 devoid of RAP has been shown to induce active Heymann nephritis,[14] and heterologous antibodies produced against preparations of gp330 devoid of RAP can induce passive Heymann nephritis.[22]

Recently acquired information about gp330 offers new ways of investigating its role in Heymann nephritis and any possible contributory role of RAP. gp330 has been shown to be a member of the low density lipoprotein receptor family,[23] and its complete primary structure has recently been determined.[24] It is composed of 4660 amino acid residues with a predicted molecular weight of 516,715. Because of its large size, it has been renamed megalin, although gp330 continues to be used. Like other members of the low density lipoprotein receptor family, gp330 is a type 1 integral membrane protein with ligand binding, growth factor and epidermal growth factor cysteine-rich repeats in the ectodomain, and NPXY sequences (involved in coated pit internalization) in the cytoplasmic domain. Recombinant gp330 proteins devoid of RAP or carbohydrate moieties can be produced, and peptides containing defined amino acid sequences can be investigated for their ability to induce disease and combine with pathogenic antibodies of Heymann nephritis. We previously reported[23] that antibodies eluted from the kidneys of rats with Heymann nephritis, induced by immunization with Fx1A, reacted with a gp330 fusion protein encoded by a 0.4-kb partial cDNA, which, based on knowledge of the complete structure of gp330,[24] can now be identified as representing 137 amino acid residues in the ectodomain, near the amino terminus. In the present study we investigated the ability of this gp330 fusion protein to induce Heymann nephritis.

Materials and Methods

Preparation of gp330-Glutathione-S-Transferase (GST) Fusion Protein

Cloning of a 0.4-kb gp330 cDNA fragment from rat kidney λgt11 cDNA library has been described previously.[23] The 0.4-kb fragment was subcloned into *Eco*RI sites of the pGEX-1 (Pharmacia Biotech, Uppsala, Sweden) expression vector. Sequence analysis showed that the fusion protein contained 137 amino acids, corresponding to residues 1114 to 1250, based on the recently published sequence of gp330.[24] Extra nucleotides were also found at the both ends of the 0.4-kb insert, which makes the in-frame construct in the pGEX-1 vector as follows: 5′GAATTC CGC TCG GAT.... .CCG ACT CCG GAATTC3′. A 0.4-kb gp330-GST fusion protein was made according to the method of Smith and Johnson.[25] Briefly, overnight cultures of bacteria transformed with 0.4-kb gp330-pGEX-1 plasmid were diluted 1:10 with fresh medium and grown for another 3 hours at 37°C before adding 1 mmol/L isopropyl β-D-thiogalactopyranoside (Sigma Chemical Co., St. Louis, MO). The cells were grown for another 4 hours, harvested by centrifugation, and resuspended in 1/50 culture volume of phosphate-buffered saline (PBS). The cells were lysed by mild sonication for 15 seconds and centrifuged at 10,000 rpm for 5 minutes at 4°C. A column was made of gluta-

thione agarose beads (Sigma) in PBS, and clear supernatant was passed through the column two times. The column was then washed thoroughly with PBS and the fusion protein was eluted with 50 mmol/L Tris-HC1 (pH 8.0) containing 5 mmol/L reduced glutathione (Sigma). Fusion protein factions were pooled and dialyzed against PBS overnight at 4°C.

The gp330-GST fusion protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)[26] with Coomassie blue staining and by Western blotting, using two previously characterized monoclonal antibodies to gp330, 14C1,[27] and 1H2.[28]

Preparation of RAP-GST Fusion Protein, GST, and RAP Protein

DH5α bacteria harboring the pGEX-39-kD expression construct were kindly provided by Dr. Joachim Herz (University of Texas Southern Medical Center, Dallas, TX).[29] The production and harvesting of RAP-GST and GST were performed as described above. RAP was cleaved from GST by thrombin (Calbiochem, San Diego, CA), as previously described.[29]

Preparation of Anti-gp330 and Anti-gp330-GST Fusion Protein Antisera

gp330 was immunoaffinity purified as described previously.[30] Briefly, a monoclonal anti-gp330 antibody designated 14C1 was coupled to Sepharose CL-4B beads (Sigma). The crude membrane extract Fx1A[5] was washed with PBS twice, then solubilized in PBS containing 1% deoxycholate (Fisher Scientific, Fairlawn, NJ), 1% Triton X-100 (Fisher), 1% polyethylene glycol compound (15 to 20 kd; Sigma) and 2 mmol/L phenylmethylsulfonyl fluoride (Sigma). The supernatant was passed through the 14C1 column. After washes, gp330 was eluted from the column with 50 mmol/L citric acid (pH 3.2; Fisher) and was neutralized with 1 mol/L Tris (pH 8.0; Fisher). gp330 was separated by 4 to 15% SDS-PAGE in the presence of 2-mercaptoethanol (Bio-Rad, Hercules, CA) and 20 mmol/L EDTA. The most prominent high molecular weight band (>440 kd) was cut out, emulsified with Freund's complete adjuvant (Sigma), and used to immunize two rabbits. Antisera from both rabbits immunoprecipitated gp330, as confirmed by reactivity with the monoclonal antibody 1H2 against gp330. The antisera showed no reactivity with the RAP-GST fusion protein, either by Western blot or enzyme-linked immunosorbent assay (ELISA). For production of antibodies against the gp330-GST fusion protein, two rabbits were immunized with 100 μg of the fusion protein in complete Freund's adjuvant. The antisera reacted with gp330, GST, and the gp330-GST fusion protein, as shown by ELISA and Western blot analysis.

Rats

Female Lewis rats weighing approximately 150 g were obtained from Charles River Laboratories (Wilmington, MA). In experiments designed to induce active Heymann nephritis, groups of rats were immunized with the gp330-GST fusion protein, with GST, or with Fx1A. The initial injections were given intradermally in Freund's complete adjuvant using 300 μg of GST-gp330 fusion protein, 200 μg of GST, or 4 mg of Fx1A. An injection of pertussis vaccine (1×10^{10} organisms) was given at a separate site. Booster injections were given at 5 and 9 weeks in incomplete Freund's adjuvant, using the initial dose of antigen. To produce passive Heymann nephritis, normal rats were injected intravenously with rabbit antisera to gp330 or to the gp330 fusion protein.

Analysis of Antibodies in Serum and Glomerular Eluates

Blood specimens were collected before immunization and at 5, 9, and 20 weeks. Glomerular eluates were prepared from renal specimens obtained at 5, 9, or 20 weeks. Pooled eluates were prepared from eight kidneys of rats immunized with gp330-GST and four kidneys of rats immunized with Fx1A. The cortices were excised, rinsed with PBS containing 2 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L *N*-ethylmaleimide, and 2 mmol/L benzamidine, and passed through 48- and 140-mesh sieves. The glomeruli were collected on a 200-mesh sieve, with extensive washes with PBS. The glomeruli were suspended for 1 hour, washed three times with distilled water containing the protease inhibitors, suspended in 20 mmol/L citric acid buffer, pH 3.2, and sonicated for 3 minutes with 40% output until no intact glomeruli were seen under the microscope. The suspensions were end-to-end rotated for 1 hour at room temperature and centrifuged at 25,000 $\times$ *g* for 30 minutes. The supernatants were collected, neutralized with 1 mol/L Tris buffer (pH 8.0), and dialyzed against PBS. The eluates were concentrated 10-fold, using negative pressure. Rat IgG concentrations were determined by ELISA.

ELISA

Sera and eluates were tested for reactivity with the gp330-GST fusion protein, with gp330, GST, and RAP by ELISA. The 96-well microtiter plates (Nunc, Naperville, IL) were coated with 5 μg/ml GST, gp330-GST, or RAP or with 1 μg/ml purified gp330 overnight at 4°C. Plates were blocked with 3% skimmed milk (Difco Laboratories, Detroit, MI). Eluates containing 1 μg/ml rat IgG or rat sera at a 1:25 dilution were added, followed by incubation with 1:500 diluted alkaline-phosphatase-conjugated goat anti-rat IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Reactions were developed with *p*-nitrophenyl phosphate (Sigma), and the absorbance was measured with the BioTek EL311 ELISA reader.

Urinalysis

Rats were housed overnight in metabolic cages, and 16-hour urine specimens were collected and analyzed for protein content by the biuret method. The normal amount of proteinuria was found to be less than 40 mg/24 hours.

Immunofluorescence Studies

Renal tissue was obtained by biopsy or nephrectomy under ether anesthesia or at sacrifice. For most studies, blocks of unfixed cortical tissue were snap-frozen in liquid nitrogen and embedded in OCT (Baxter, Medford, MA). Sections were cut at 4 μm, air dried, and fixed briefly in acetone. A few specimens were obtained after renal perfusion with paraformaldehyde-lysine-periodate (PLP).[17] Rats were perfused via the aorta under pentobarbital anesthesia with 150 ml of Hank's buffer followed by the same amount of PLP fixative. The kidneys were removed and cut into 5-mm slices, which were immersed in PLP fixative buffer at 4°C overnight and then in 30% sucrose before freezing in liquid nitrogen. For immunofluorescence studies, sections were cut at 4 μm, air dried for 30 minutes, and stained as described.[31]

Antibodies Used in Immunofluorescence Studies

For detection of rat C3 and IgG, fluorescein isothiocyanate (FITC)-labeled anti-C3 (Cappel Laboratories, Durham, CA) or anti-rat IgG (Sigma) antibodies were used.

Other antigens were detected by indirect immunofluorescence using the following primary antibodies. Rabbit anti-gp330 and anti-gp330-GST fusion protein antisera were prepared as described above. Two murine monoclonal anti-gp330 antibodies (14C1 and 1H2) and a rabbit antiserum to a peptide sequence of the cytoplasmic domain of gp330 have been characterized and described previously.[27,28] Rabbit anti-GST antiserum was obtained from Amrad Corp. (Victoria, Australia). Rat immunoglobulin isotypes were detected by use of the immunoglobulin isotyping kit (The Binding Site, San Diego, CA), containing IgG fractions of sheep anti-rat IgA, IgG1, IgG2a, IgG2b, IgG2c, and IgM. Rat C6 and C7 were detected with goat anti-human C6 antisera and a murine monoclonal antibody against human C7, both of which have been shown to cross-react with the corresponding rat components (Quidel, San Diego, CA).

The following secondary antibodies were used: rabbit anti-goat IgG (Cappel), goat anti-rabbit IgG (Sigma), rabbit anti-mouse IgG (Sigma), and donkey anti-sheep IgG (The Binding Site), all of which were FITC labeled.

As controls for immunofluorescence staining, primary antibodies were omitted or preimmune sera were used and sections were incubated with the secondary antibodies; in no case was staining of glomerular deposits seen.

Results

Characterization of the gp330-GST Fusion Protein

As shown in Figure 1, the Gp330-GST fusion protein reacted with one of two monoclonal anti-gp330 antibodies. The estimated molecular mass of the protein coincided with the predicted value of approximately 40 kd.

Two types of studies were performed to determine whether the gp330-GST fusion protein reacted with RAP. In one approach, the RAP-GST protein was subjected to SDS-PAGE and transferred to nitrocellulose, which was blocked with 5% skimmed milk in PBS/Tween-20, followed by incubation with the gp330 fusion protein for 1 hour. The blots were then incubated with the monoclonal gp330 antibody 1H2; no reactivity was found. In a second approach, we attempted to demonstrate binding of the gp330-GST fusion protein to cells in frozen sections of liver and kidney, where RAP is abundant. Sections were incubated with the gp330-GST fusion protein and then with rabbit anti-GST antibodies followed by FITC-labeled goat anti-rabbit IgG (a technique that has

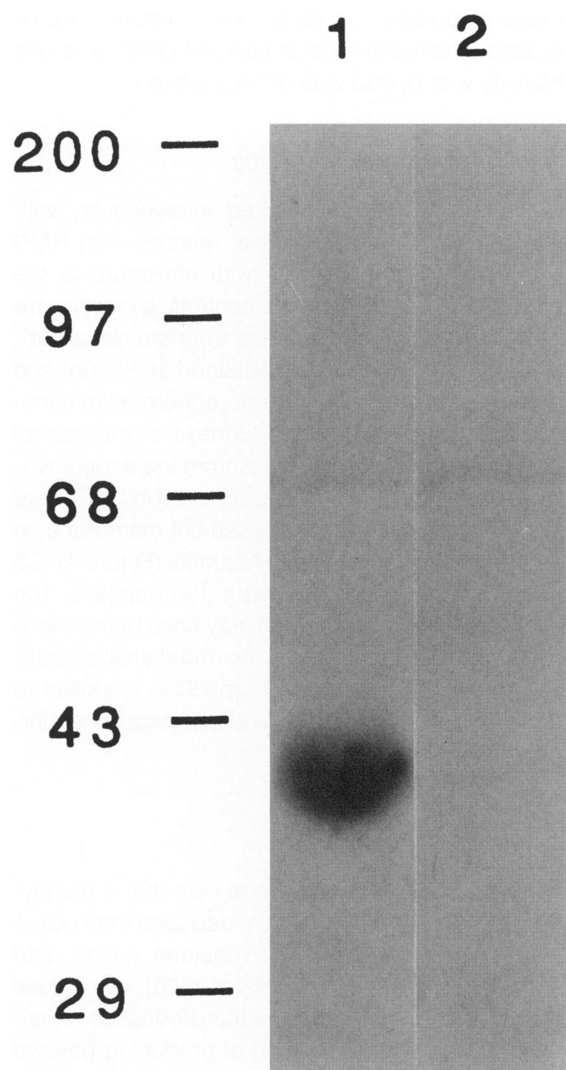


Figure 1. *Western blot analysis of the gp330-GST fusion protein after SDS-PAGE and transfer to nitrocellulose. The protein appears at a band at approximately 40 kd, as identified by the monoclonal anti-gp330 antibody 1H2* (lane 1). *There is no reactivity with another monoclonal anti-gp330 antibody, 14C1* (lane 2). *Standard molecular weight markers are shown at the left.*

been shown to detect bound GST fusion proteins).[32] No staining was detected, indicating lack of binding to RAP.

Active Heymann Nephritis

Three groups of rats were studied, consisting of four rats immunized with Fx1A, eight rats immunized with the gp330-GST fusion protein, and seven rats immunized with GST. One of the four rats immunized with Fx1A developed abnormal proteinuria (90 mg/24 hours), whereas all of the other rats had urinary protein excretion within the normal range.

All of the rats immunized with Fx1A or with the gp330-GST fusion protein, but none of the rats injected with GST, developed deposits of Ig along the glomerular basement membrane in a pattern typical of Heymann nephritis (Figure 2). The deposits were uniformly stained by antibodies to IgG subclasses IgG1, IgG2a, and IgG2b (but not IgG2c) and to IgA; only focal staining for IgM was seen. In both groups the deposits appeared to be more prominent at 20 weeks than at 5 weeks. At all times the deposits in the Fx1A group were larger and stained more intensely for IgG and gp330 than in the rats immunized with the gp330 fusion protein. The more intense staining for gp330 seen in the Fx1A-injected rats may reflect a greater production of gp330 by podocytes, a process that has recently been shown to occur in active Heymann nephritis.[33] Furthermore, complement components (C3, C6, and C7) were found in deposits only in Fx1A-injected animals. The two groups also differed with respect to tubular changes; although both Fx1A- and gp330-fusion-protein-injected rats showed brush border staining for IgG, this was generally more intense in Fx1A-injected rats, and only these contained debris, which stained for IgG and gp330, in the lumen of some tubules. Glomerular deposits in both Fx1A- and gp330-fusion-protein-injected rats were intensely stained by polyclonal and monoclonal anti-gp330 antibodies known to be reactive with epitopes in the ectodomain.[28] In contrast, an antiserum against a cytoplasmic domain peptide of gp330 failed to stain.[28] One of the monoclonal antibodies (14C1) that produced staining of the deposits did not react with the gp330 fusion protein in Western blots. However, as shown previously,[27] 14C1 reacts with intact gp330 subjected to SDS-PAGE and Western blotting, which indicates that it recognizes epitopes dependent on primary structure rather than on conformational epitopes that might be lacking in the gp330 fusion protein. A rabbit antiserum against the fusion protein resulted in intense staining of deposits in Fx1A-injected rats and somewhat less intense staining in rats injected with the gp330 fusion protein (Figure 2D), paralleling the results obtained with the other anti-gp330 antibodies.

The glomerular deposits in the Fx1A- and gp330-GST-injected rats showed faint staining for RAP in fresh frozen sections, but not in sections prepared from aldehyde (PLP)-fixed tissue. We considered the possibility that the staining resulted from artifactual redistribution of RAP. In a previous study[17] we showed that, in sections prepared from fresh frozen normal rat kidneys, RAP is released from its normal intracellular location, after which it combines with

gp330 on proximal tubule brush borders. In contrast, in aldehyde-fixed tissue, we found staining for RAP to be entirely intracellular, which reflects its normal location. To determine whether exogenously applied RAP binds to gp330 in glomerular deposits, we incubated fresh frozen kidney sections from rats immunized with Fx1A or the gp330 fusion protein with a RAP-GST fusion protein, which was detected by immunofluorescence using anti-GST antibodies. Faint to moderate staining was found in the deposits (and intense staining was seen in brush border regions of proximal tubules; Figure 3a). Control sections incubated with GST followed by anti-GST antibodies showed no staining of deposits or brush border regions (Figure 3b). The results indicate that soluble RAP can bind to gp330 in glomerular deposits, and they support the interpretation that the staining for RAP seen in frozen sections resulted from artifactual redistribution but do not entirely exclude the possibility that some RAP is co-shed with gp330 from podocytes.

Antibody Specificities in Sera and Glomerular Eluates of Rats Immunized with the gp330 Fusion Protein or Fx1A

The results of ELISAs to measure antibodies reactive with purified gp330, the gp330-GST fusion protein, and RAP are shown in Tables 1 and 2. In both serum and glomerular eluates, roughly comparable concentrations of antibodies against purified gp330 were found in rats immunized either with the gp330 fusion protein or with Fx1A. In contrast, antibody levels against the gp330 fusion protein were considerably higher in rats immunized with the fusion protein. As shown in Table 1, some of the serum antibodies in rats immunized with the fusion protein were directed against GST.

No serum antibodies against RAP were found. However, glomerular eluates from gp330-GST-immunized rats did show low levels of reactivity with RAP (Table 2). In the absence of serum antibodies, it seemed unlikely that this was due to anti-RAP antibodies. We considered an alternative possibility, namely that reactivity resulted from undissociated immune complexes containing gp330 (to which RAP bound) rather than from antibodies to RAP. To investigate this, we performed ELISAs in the presence of EDTA, which eliminates calcium-dependent binding of gp330 to RAP.[12,19] As shown in Table 2, EDTA abolished the reactivity of eluates with RAP, whereas reactivity with gp330 was not decreased.

Passive Heymann Nephritis

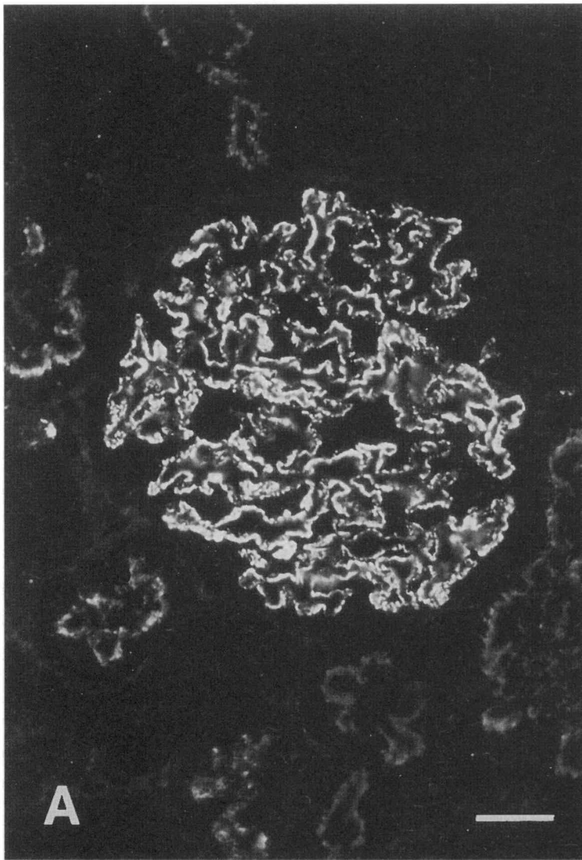
Two normal rats were injected intravenously with rabbit antiserum to gp330 (not reactive with RAP) (1 ml per rat) and two rats with antiserum to the gp330-GST fusion protein. As controls, two rats were injected with preimmune serum from the donor rabbits. Renal specimens were obtained at 2 hours and at 1 and 5 days. There were no abnormal immunofluorescence findings in the kidneys of the control rats. However, in all specimens from the experimental groups, fine granular deposits of rabbit IgG were found along the glomerular basement membrane, in a pattern typical of Heymann nephritis (Figure 4). C3 and gp330 were not detected in the deposits. The absence of staining for gp330 may have been due to blocking of antigenic sites by the rabbit antibodies or lack of sufficient amounts of gp330 in deposits to permit its detection. None of rats developed abnormal proteinuria.

Discussion

In the present study we have shown that a nonglycosylated gp330 fusion protein, deduced from cDNA encoding gp330/megalin[24] to include amino acid residues 1114 to 1250 (of a total of 4660), can induce active Heymann nephritis and the production of heterologous antibodies capable of producing passive Heymann nephritis. These results define at least one region in the ectodomain of gp330, toward the amino terminus, that contains peptide sequences capable of stimulating specific helper T cells leading to an anti-gp330 autoantibody response. However, the epitopes on native gp330 recognized by the autoantibodies induced either with the fusion protein or with Fx1A (which contains the entire gp330 molecule) have not been characterized, and it is therefore not known to what extent the spectrum of autoantibodies stimulated by these immunogens differ or overlap.

Certain findings indicate that the glomerular deposits in rats immunized with the gp330 fusion protein included parts of the ectodomain of gp330 other than the region of the fusion protein. Thus, a monoclonal anti-gp330 antibody (14C1) stained the de-

Figure 2. *Immunofluorescence findings at 20 weeks in the kidney of a rat immunized with the gp330-GST fusion protein. The sections were stained by direct immunofluorescence with goat antibodies to rat IgG* (A), *by indirect immunofluorescence with monoclonal anti-gp330 antibodies 1H2 or 14C1* (B *and* C, *respectively*), *or with rabbit antiserum to the gp330-GST fusion protein* (D). *All show granular staining along the glomerular basement membrane in a pattern typical of Heymann nephritis, as well as focal* (A) *or diffuse* (B *to* D) *apical staining of proximal tubule cells. Bar, 28 μm.*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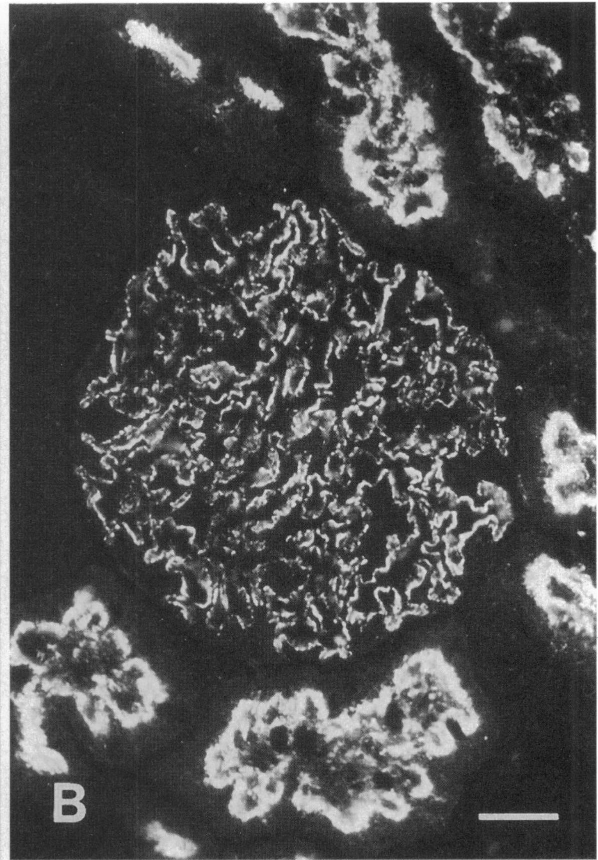
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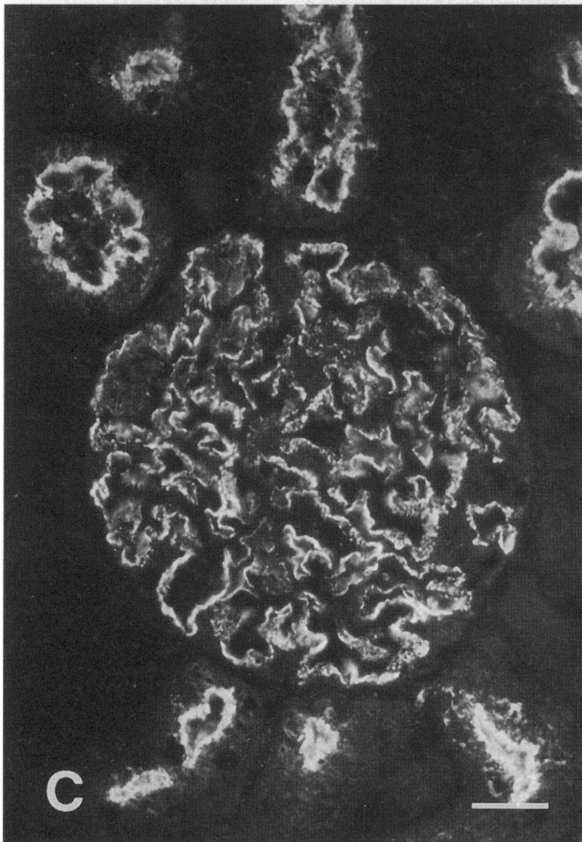
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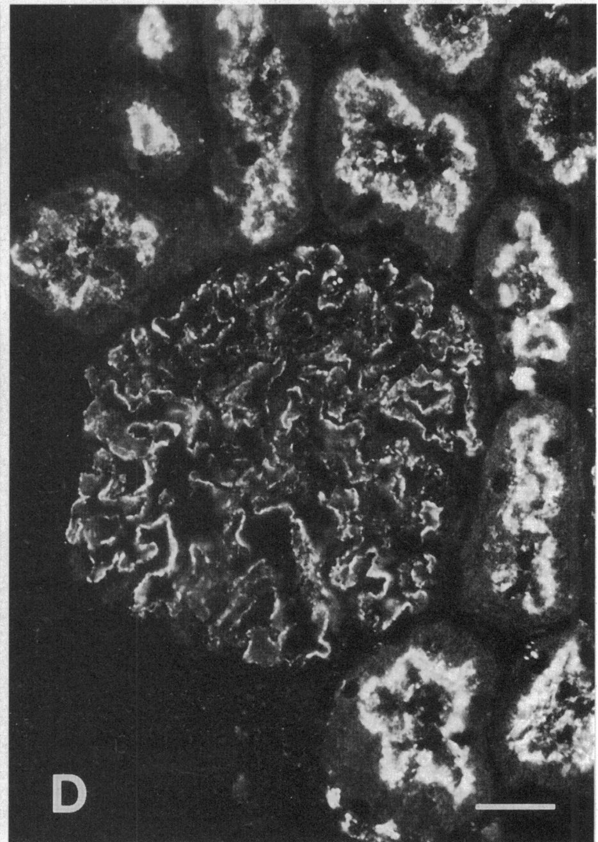
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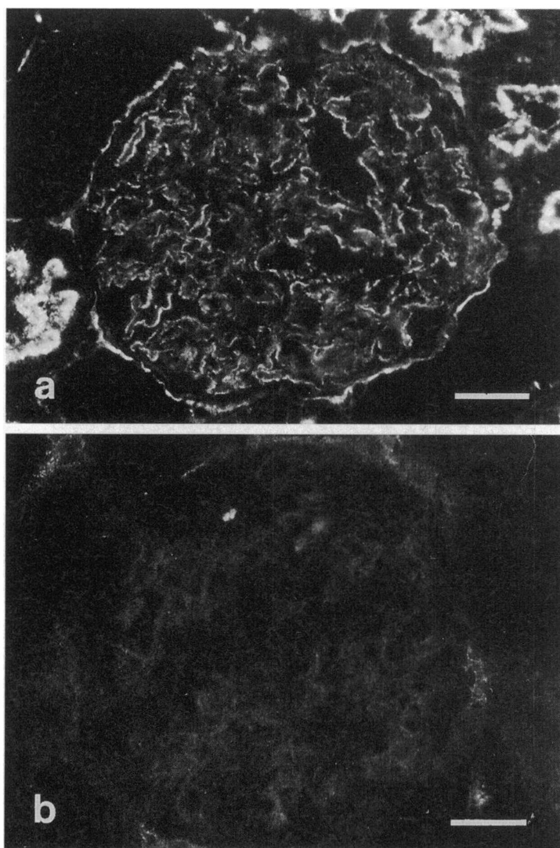

Figure 3. *Kidney from a rat immunized with the gp330-GST fusion protein.* **a:** *The frozen section was incubated with RAP-GST followed by rabbit anti-GST antibodies and then FITC-labeled goat anti-rabbit IgG. There is granular staining along glomerular capillary walls, consistent with the location of deposits, as well as staining of parietal cells and proximal tubule brush borders.* **b:** *The section, which was first incubated with GST rather than RAP-GST, shows no staining. Bar, 28 μm.*

posits, even though it did not react with the fusion protein *in vitro*. In addition, we showed that exogenously applied RAP could combine with gp330 in the deposits but did not react with the gp330 fusion protein *in vitro*.

Two general types of explanation can be invoked to explain how other portions of gp330 could be present in the deposits of rats immunized with the gp330 fusion protein. First, the autoantibodies induced by the fusion protein may react only with epitopes in the region spanning amino acids 1114 to 1250 and yet, perhaps by producing a conformational change in gp330, expose a cleavage site closer to the transmembrane domain[34] or release of a form of gp330 that lacks a cytoplasmic domain.[28] Second, the fusion protein may induce the formation of autoantibodies against epitopes in other regions of the molecule, including sites where cleavage occurs.

In confirmation of a recent report,[22] the present results show that heterologous anti-gp330 antibodies can induce glomerular deposits of passive Heymann nephritis without the participation of antibodies to RAP. In that study,[22] antibodies eluted from glomeruli were shown to react with two proteolytic fragments (140 and 75 kd) of gp330, but the location of these fragments within gp330 was not determined. Our results also show that a RAP-gp330 complex is not required for the induction of pathogenic anti-gp330 autoantibodies. Even the remote possibility that the gp330 fusion protein forms an immunogenic complex with RAP *in vivo* (which might occur at the injection site, as RAP is present in macrophages and fibroblasts)[32] seems to be excluded, in view of the lack of binding of the fusion protein to RAP *in vitro*.

Nevertheless, complexes produced by antibodies to gp330 might include RAP, if this molecule is combined with gp330 on the podocyte surface and is co-shed into deposits. To evaluate this possibility we studied kidney sections by immunofluorescence for the presence of RAP in deposits. Although we detected staining for RAP, we obtained evidence that this could be accounted for by release of intracellular RAP during tissue processing followed by binding of RAP to gp330 in deposits.

As in most other reports on Heymann nephritis induced with purified preparations of gp330 (or antibodies to gp330), the glomerular deposits produced with the gp330 fusion protein were rather small and did not stain for complement components C3, C6, or C7, and abnormal proteinuria was not

Table 1. *Reactivity of Serum IgG Antibodies (ELISA OD Absorbance)*

	Preparation used for immunization								
	gp330-GST			GST			Fx1A		
Protein tested	5 weeks (n = 8)	9 weeks (n = 7)	20 weeks (n = 6)	5 weeks (n = 7)	9 weeks (n = 7)	20 weeks (n = 5)	5 weeks (n = 4)	9 weeks (n = 3)	20 weeks (n = 2)
gp330	0.561 ± 0.067	0.666 ± 0.060	0.712 ± 0.061	0.049 ± 0.034	0.054 ± 0.038	0.044 ± 0.050	0.461 ± 0.124	0.529 ± 0.070	0.326 ± 0.000
gp330-GST	0.816 ± 0.030	0.729 ± 0.033	0.767 ± 0.082	0.567 ± 0.104	0.777 ± 0.044	0.793 ± 0.013	0.042 ± 0.011	0.088 ± 0.081	0.046 ± 0.000
GST	0.321 ± 0.095	0.547 ± 0.049	0.592 ± 0.019	0.441 ± 0.071	0.613 ± 0.093	0.675 ± 0.117	0.006 ± 0.003	0.005 ± 0.004	0.004 ± 0.000
RAP	0.091 ± 0.009	0.011 ± 0.003	0.037 ± 0.009	0.002 ± 0.001	0.012 ± 0.002	0.035 ± 0.007	0.004 ± 0.002	0.009 ± 0.003	0.006 ± 0.000

Values represent the average of results of all serum specimens (1:25 dilution) tested. Specimens from at least one-half of the rats were tested at each time indicated. The decrease in number of rats over time resulted from deaths due to anesthesia, administered before nephrectomy, or blood collection or from sacrifice to obtain renal tissue.

Table 2. *Reactivity of IgG in Pooled Glomerular Eluates (ELISA OD Absorbance)*

Protein tested	Source of IgG					
	Eluates from gp330-GST-immunized rats		Eluates from Fx1A-immunized rats		Normal rat serum IgG	
	No EDTA	EDTA	No EDTA	EDTA	No EDTA	EDTA
gp330	0.420 ± 0.026	0.556 ± 0.076	0.589 ± 0.025	0.520 ± 0.048	0.087 ± 0.006	0.084 ± 0.014
gp330-GST	0.505 ± 0.007	0.568 ± 0.053	0.181 ± 0.002*	0.106 ± 0.005*	0.069 ± 0.007	0.028 ± 0.002
GST	0.085 ± 0.022†	0.108 ± 0.017*	0.044 ± 0.004	0.035 ± 0.004	0.037 ± 0.002	0.025 ± 0.002
RAP	0.198 ± 0.010*	0.062 ± 0.014‡	0.098 ± 0.011‡	0.041 ± 0.009‡	0.075 ± 0.023	0.038 ± 0.008

As a control for reactivity of IgG in glomerular eluates, normal rat IgG was used (column 3); all preparations were adjusted to a concentration of 1 μg/ml.
*Values are significantly higher than normal rat serum IgG ($P < 0.01$).
†Value is significantly higher than normal rat serum IgG ($P < 0.05$).
‡Values do not differ significantly from normal rat serum IgG ($P > 0.05$).

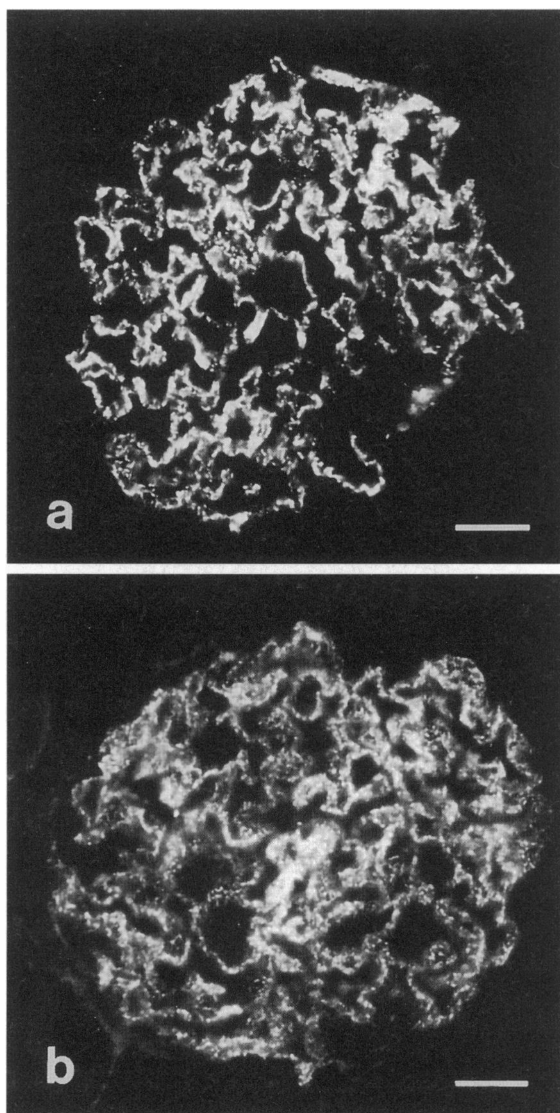

Figure 4. *Immunofluorescence finding in two rats 5 days after administration of rabbit antiserum against gp330* (a) *or against the gp330 fusion protein* (b). *In each case there is bright granular staining for rabbit IgG along the glomerular basement membrane. Bar, 20 μm.*

seen. Proteinuria appears to require a critical amount of immune deposits[35,36] and/or complement fixation.[10] The factors required for activation of complement in Heymann nephritis have not been entirely defined, but in the passive model, Susani et al[8] have obtained evidence that a combination of antibodies to gp330 and to a renal glycolipid is needed. There is no evidence that antibodies to RAP contribute to complement activation. It is not clear why immune deposits containing gp330 sometimes fail to activate complement, as the antigen is multivalent and as the deposits contain classes of IgG capable of fixing complement,[6,35] as confirmed in the present study.

In summary, we have identified an immunogenic region of gp330 capable of inducing nephritogenic antibodies. Additional studies are needed to define other immunogenic regions of gp330 and to identify the epitopes recognized by pathogenic antibodies.

Added Note

The sequence of the gp330 fusion protein we used is in the same region of the molecule as that found by Saito et al[37] to be recognized by pathogenic heterologous antibodies to gp330/megalin.

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