gp330 associates with a 44-kDa protein in the rat kidney to form the Heymann nephritis antigenic complex

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Contributed by Marilyn Gist Farquhar, March 26, 1992

ABSTRACT Using antibodies isolated from glomeruli of nephritic rats we have previously identified a 330-kDa cell surface glycoprotein (gp330) as a major pathogenic antigen of Heymann nephritis (HN), an experimental model of human membranous glomerulonephritis. Recently, we have isolated a cDNA clone, C14, encoding Heymann disease.

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immunoreactive with antibodies to the C14 protein (C14p) and α2-MRAP.

MATERIALS AND METHODS

Antibodies. Polyclonal antiserum to gp330 and C14/β-galactosidase fusion protein were prepared as described (6, 9). Anti serum to rat α2-MRAP/glutathione transferase fusion protein was a generous gift from Joachim Herz (University of Texas Southwestern Medical Center, Dallas, TX). Anti-gp330 monoclonal antibody (mAb) 20B was prepared against a Triton X-114 extract of glomerular cell membranes (12), and mAb D1-55F2 was raised against purified gp330 (6).

Kidney Microvilli. Microvillus membranes were prepared as described (6) and stored in liquid nitrogen.

Protein Iodinations. Iodinations were performed using the Iodo-Gen method (Pierce) (13). Labeled protein was separated from free 125I on a PD-10 column (Pharmacia) preequilibrated with phosphate-buffered saline (PBS) containing 0.1% Triton X-100. Specific activities for all iodinations were routinely 20,000–50,000 cpn/ng of protein.

Immunoprecipitations. Polyclonal antiserum or ascites fluid (diluted 1:200) was preincubated with protein A-agarose (Bio-Rad) or goat anti-mouse agarose (Sigma), respectively, for 2 h at 23°C. The beads were washed twice with PTO (PBS/0.5% Tween 20/5 mg of ovalbumin per ml) and incubated with radiolabeled brush border extract (107 cpm) for 16 h at 4°C. They were then washed twice with RIPA buffer (10 mM Tris, pH 7.2/150 mM NaCl/1% deoxycholate/1% Triton X-100/0.1% sodium dodecyl sulfate/1% aprotinin/2 mM phenylmethanesulfonyl fluoride), twice with PTO, and twice with PT (PBS/0.5% Tween 20). Laemmli sample buffer was added to the beads with 2% 2-mercaptoethanol (Bio-Rad) and boiled for 5 min. Immunoprecipitates were separated by SDS/PAGE on 5–12% gradient gels.

Affinity Purification of gp330. IgG was purified from 20B ascites fluid and coupled to Affi-Gel 10 beads (Bio-Rad). Kidney microvillar extract was diluted 10-fold with 20 mM Hepes, pH 7.4/150 mM NaCl/2 mM CaCl2/1% Triton X-100 and recirculated over the 20B affinity column for 16 h at 4°C. The column was washed with 20 column volumes of dilution buffer and eluted with 10 mM sodium acetate, pH 3.0/5 mM EDTA/1% Triton X-100 into 1-ml fractions. Eluant was neutralized by collecting directly into 200 μl of 3 M Tris buffer (pH 8.8). Peak fractions, determined by SDS/PAGE, were pooled, dialyzed exhaustively against PBS containing 0.1% acetic acid.

Abbreviations: α2-MRAP, α2-macroglobulin receptor-associated protein; LDL, low density lipoprotein; LRP, LDL receptor-related protein; C14, clone 14; C14p, C14 protein; HN, Heymann nephritis; mAb, monoclonal antibody; ER, endoplasmic reticulum; PLP, periodate-lysine/paraformaldehyde.

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**Heymann nephritis (HN) is an autoimmune disorder that provides an animal model for the study of human membranous glomerulonephritis (1, 2). The disease is initiated by the binding of circulating antibodies to glomeruli (3, 4), where they interact with antigens at the surface of the glomerular epithelium (5, 6). The resultant immune complexes are then shed and crosslinked to the basement membrane, resulting in severe glomerular injury and proteinuria (7). Antibodies eluted from glomeruli of HN rats demonstrate specificity for gp330, a large membrane-associated glycoprotein (5). gp330 is localized in coated pits found in the proximal tubule brush border and at the base of the foot processes of glomerular podocytes (6). The mechanism that initiates immune complex deposition is not understood but has been thought to involve the presentation of pathogenic epitopes present within gp330 (7, 8). Using antibodies eluted from the glomeruli of HN rats, Pietromonaco et al. (9) identified a cDNA, clone 14 (C14), encoding an ≈35-kDa protein that contains a major pathogenic domain of the HN antigen. Two other proteins—human α2-macroglobulin receptor-associated protein (α2-MRAP) (10) and a mouse heparin binding protein (HBP-44) (11)—were subsequently shown to possess a high degree of homology to the predicted amino acid sequence of the C14 cDNA. In this report, we show that gp330 forms a stable heterodimeric complex with a 44-kDa polypeptide that demonstrates immunoreactivity with antibodies to the C14 protein (C14p) and α2-MRAP.**

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Triton X-100, and concentrated with Aquacide II (Calbiochem). Protein concentration was determined by the bicinchoninic acid assay (Pierce).

**Oligomerization Assay.** The oligomerization assay was performed on newborn rat kidneys biosynthetically labeled as described (unpublished work). Briefly, kidneys were removed from 1- to 2-day-old rats, injected with RPMI medium (Irvine Scientific) containing 3 mg of collagenase per ml (Worthington), and gassed with 95% O2/5% CO2. The tissue was manually dissociated with forceps, passed through a Pasteur pipette 20 times, washed in methionine-free RPMI medium, and incubated with 500 μCi of Tran35S-label (ICN; 1 Ci = 35 GBq) for 3 hr at 37°C with constant gassing. The tissue was washed in ice-cold TBS (50 mM Tris, pH 7.4/150 mM NaCl/1 mM disopropyl fluorophosphatate), disrupted with a Dounce homogenizer. The resulting homogenate was centrifuged at 750 × g for 10 min, after which the supernatant was centrifuged at 100,000 × g for 1 hr at 4°C. The pellet was resuspended in lysis buffer (20 mM Mes/30 mM Tris, pH 7.4/150 mM NaCl/1% Triton X-100).

Velocity gradient centrifugation was performed as described (14). Briefly, insoluble material was removed by centrifugation at 15,000 × g for 15 min at 4°C. The supernatant was overlaid onto a linear 5–25% sucrose gradient and centrifuged at 40,000 rpm for 4 hr at 4°C in a Beckman SW41 rotor. Fractions were collected and immunoprecipitates were prepared using 20B ascites fluid (1 hr at room temperature) and a rabbit anti-mouse IgG bridge (1 hr) prior to addition of protein A beads. Sedimentation coefficients were calculated using protein standards, and sucrose density was determined from the refractive index.

**Electrophoresis and Immunoblotting.** Proteins were separated by SDS/PAGE (15). For immunoblotting, proteins were transferred to Immobilon (Millipore) using a semi-dry blotting apparatus (Millipore). Blots were blocked for 16 hr at 4°C with 50 mM Tris, pH 7.4/150 mM NaCl/3% bovine serum albumin (Sigma), followed by a 2-hr incubation at room temperature with the indicated antiserum. They were then washed 10 min each as follows: twice with 50 mM Tris, pH 7.4/150 mM NaCl (Tris/NaCl), twice with Tris/NaCl plus 0.05% Nonidet P-40, twice with Tris/NaCl. The Immobilon membranes were incubated for 1.5 hr at 23°C with 1 × 10^5 cpm of [125I]-labeled protein G per ml (Sigma) diluted into blocking buffer, washed as described above, dried, and exposed to x-ray film at −70°C.

**Immunocytochemistry.** For immunofluorescence aceton-dipped cryostat sections of unfixed kidney and semithin (2 μm) cryosections of periodate/lysin/paraformaldehyde (PLP)-fixed kidney, prepared as described (16), were incubated with rabbit anti-C14p IgG (10 μg/ml) followed by incubation with rhodamine-conjugated anti-rabbit F(ab')2 (diluted 1:100) (Tago). For immunoelectron microscopy, sections of PLP-fixed kidneys were incubated in anti-C14p IgG followed by peroxidase-conjugated goat anti-rabbit Fab (Biosys) and processed for immunoperoxidase (6, 7).

**RESULTS**

**gp330 Associates with a 44-kDa Polypeptide in Kidney Brush Border.** Immunocytochemical and immunoblotting data indicate that gp330 is an abundant protein in the brush border of rat kidney proximal tubule (6, 16). To facilitate biochemical characterization of gp330, microvillar fractions were prepared from perfused rat kidneys to enrich for membrane proteins associated with the brush border (Fig. 1, lane B). The membranes were solubilized in 1% Triton X-100, radioiodinated, and immunoprecipitated using a gp330-specific polyclonal antibody (6). Fig. 1 (lane C, arrows) demonstrates that under these conditions the polyclonal antiserum coprecipitated two polypeptides with apparent molecular masses of 450 and 44 kDa. The apparent molecular mass of the larger polypeptide, relative to the laminin heavy chain (400 kDa under reducing conditions), is greater than previously reported for gp330 using different gel systems and standards (5).

To exclude the possibility of cross-reactivity of the polyclonal antiserum with the 450- and 44-kDa species, immunoprecipitations were performed with two previously characterized mAbs, 20B and D1-55F21, specific for gp330 (6, 12). Both coprecipitated the 450- and 44-kDa polypeptides (lanes D and E). These data indicate that gp330 forms a heterodimeric complex with a 44-kDa polypeptide in the microvillar fraction derived from the proximal tubule brush border.

**Antibodies Eluted from Glomeruli of RN Rats and Anti-C14p Antibodies Precipitate gp330 and the 44-kDa Protein.** To further investigate the nature of the gp330–44-kDa dimer, we next performed immunoprecipitation studies with (i) IgG eluted from the glomeruli of rats with passive HN induced by injection with anti-gp330 antibodies or (ii) antiserum raised against a fusion protein (C14p) previously shown to bind to glomeruli in vivo and induce passive HN (9). Both antibodies precipitated the 450- and 44-kDa polypeptides under nonde-naturing conditions (Fig. 1, lanes F and G). The larger protein was previously shown to be gp330 by immunoprecipitation (5, 6, 16) and Western blot analysis (17). These results suggest that the HN antigen is a dimer consisting of gp330 and an associated 44-kDa subunit.

The antibodies eluted from the glomeruli of RN rats immunoprecipitated both subunits from Triton X-100-solubilized radioiodinated brush border proteins in relative stoichiometric amounts, as determined by densitometry and predicted tyrosine content. However, anti-C14p immunoprecipitated predominantly the 44-kDa polypeptide, suggesting that some of the protein is present dissociated from gp330 in the microvillar fraction. In addition, a 32-kDa protein was coprecipitated with C14p antiserum (Fig. 1, lane G). It is not clear whether this polypeptide represents a degradation product of the 44-kDa polypeptide, a cross-reactive product with the antibody, or another subunit of the complex.

**The 44-kDa Polypeptide Associated with gp330 Is Immunologically Identical to a2-MRAP.** Because of the 73% amino acid sequence homology between the human a2-MRAP and the predicted amino acid sequence of C14p (10), we investigated the possibility that the small subunit of the gp330–44-kDa complex may correspond to the rat a2-MRAP. Immu-
noprecipitations were prepared in parallel from radioiodinated brush border using antisera to a C14 fusion protein and a fusion protein containing the rat α2-MRAP sequence (Fig. 2A, lanes 1 and 2). Both antisera precipitate ~450- and 44-kDa polypeptides with identical electrophoretic mobilities. To confirm that the antibodies are specific for the 44-kDa subunit, the gp330-44-kDa complex was purified on an anti-gp330 mAb 20B affinity column (Fig. 2B) and subjected to Western blot analysis using C14p (Fig. 2C, lane 1) or rat α2-MRAP (Fig. 2C, lane 2) antisera. Both antisera demonstrated immunoreactivity toward the 44-kDa polypeptide. Taken together, these data strongly suggest that the 44-kDa subunit shares immunological identity with C14p and α2-MRAP.

gp330 Forms a Stable Association with the 44-kDa Protein. Cosedimentation during velocity gradient centrifugation provides a qualitative assay of protein subunit assembly and oligomerization (18). To verify and extend the immunoprecipitation data demonstrating the existence of a gp330-44-kDa complex, biosynthetically labeled baby rat kidney tissue was prepared, solubilized with Triton X-100, and fractionated on a 5-25% linear sucrose gradient. The resulting fractions were immunoprecipitated with anti-gp330 mAb 20B (Fig. 3). gp330 and the 44-kDa protein cosedimented and were coprecipitated across several fractions with a peak corresponding to a sedimentation coefficient of 19.3 S, based on bovine thyroglobulin (669 kDa) as a standard. In addition, mAb 20B precipitates gp330 from gradient fractions >19.3 S. These results indicate that gp330 forms a heterooligomeric complex with the 44-kDa protein that is resistant to nonionic detergent extraction and stable to long-term centrifugation. Further, under nonreducing conditions, gp330 forms high molecular mass multimers resolved by SDS/PAGE, suggesting possible disulfide bond formation during the oligomerization process (Fig. 2B, lane 3). gp330 also migrated more slowly under reducing conditions, indicating the presence of intrachain disulfide bridges (Fig. 2B, lane 2). The identity of the 50-kDa protein that cosediments with the gp330-44-kDa complex is not known, but it may be an additional subunit of the multimeric complex.

**DISCUSSION**

In this report, we have demonstrated that the HN antigen is represented within a multimeric complex consisting of two subunits—a large subunit with an apparent molecular mass that varies from 330 to 450 kDa (depending on the gel system) and a smaller, ~44-kDa subunit. The larger polypeptide was previously identified as gp330, a glycoprotein present in coated pits of the kidney brush border and in the foot processes of glomerular podocytes (6, 7). Our present data show that the 44-kDa subunit (i) is specifically recognized by antisera raised against a fusion protein encoded by the C14 cDNA (J. Herz, M. S. Brown, and J. L. Goldstein, personal communication). C14p/α2-MRAP forms an association with gp330 that is stable to mild detergent extraction and long-term centrifugation, indicating a relatively high-affinity interac-

**Immunocytological Localization of C14p/α2-MRAP.** By indirect immunofluorescence on acetone-fixed cryostat sections of rat kidney, the anti-C14p IgG labeled the brush border region of proximal tubules, in particular the basal region of the microvilli (Fig. 4A). The pattern was similar to that seen with anti-gp330 IgG (5, 7). Similar labeling was obtained with anti-α2-MRAP IgG (data not shown). In glomeruli a very faint diffuse granular labeling was obtained with anti-C14p IgG (Fig. 5A).

By contrast, in tissues fixed in PLP, the staining pattern obtained with anti-C14p IgG was very different. There was no staining of the brush border of proximal tubules; instead, a reticular, apical cytoplasmic immunofluorescence was seen (Fig. 4B). By immunoelectron microscopy (Fig. 4D), diamobenzidine reaction product was found only in the cisternae of the ER. Similar staining of the ER was also found in glomeruli (Fig. 5 B–D). This suggests that the 44-kDa protein contains two or more epitopes, one that is demonstrable at the cell surface only in the native protein and another that is detectable in the rough ER primarily in fixed tissue.

![Fig. 2.](image)

**Fig. 2.** The 44-kDa protein associated with gp330 is immunologically identical to α2-MRAP and C14p. (A) Immunoprecipitates prepared in parallel from freshly isolated and radiolabeled renal microvillar proteins with C14p antiserum (lane 1) or α2-MRAP antiserum (lane 2) demonstrating coprecipitation and comigration of the large and small subunits. (B) gp330-44-kDa complex purified by affinity chromatography on an anti-gp330 mAb (20B) column was separated by SDS/PAGE under reducing (lane 2) or nonreducing (lane 3) conditions. Lane 1, molecular mass standards (in kDa). (C) Immunoblot analysis of purified gp330-44-kDa complex using a 1:500 dilution of either anti-C14p (lane 1) or anti-α2-MRAP (lane 2). Both antisera show immunoreactivity specific for the 44-kDa subunit.
function in the binding blot ligand may common subunit (21). A receptor (20), suggesting tubules, revealed homologies to the receptor (19). The functional significance of receptor anti-C14p IgG. Oxidase localization of cytoplasmic staining acetone-fixed lipoprotein to the receptor (19). The functional significance of receptor anti-C14p IgG. Oxidase localization of cytoplasmic staining acetone-fixed cryostat sections from normal rat kidney incubated with anti-C14p IgG. Apical labeling is seen at the base of the microvilli (MV) of the proximal tubules (arrowheads). (B and C) Similar immunofluorescence preparation of a PLP-fixed kidney section incubated with anti-C14p IgG. Staining is concentrated in the apical cytoplasm. The microvilli (MV) whose position is indicated in the phase-contrast micrograph (C) are unstained. (D) Immunoelectron microscopy revealed that the apical staining seen in the proximal tubule in fixed tissues is due to the presence of the 44-kDa protein in the rough endoplasmic reticulum (ER). L, lumen; N, nucleus; CP, coated pit; MVB, multivesicular body. (A, ×500; B and C, ×400; D, ×18,200.)

The physiological function of gp330 is currently unknown, but its localization in coated pits (6, 16, 22) suggests that it may serve as a cell surface receptor. Based on Western and ligand blot analysis, plasminogen has recently been identified as a potential ligand for gp330 (23). In rat kidney proximal tubules, gp330 is found exclusively in coated pits located at the base of brush border microvilli (16) that are known to function in endocytosis (24, 25). Immunoprecipitation studies performed on brush border membranes purified from the adult rat kidney demonstrate that the 44-kDa subunit may be present dissociated from gp330 in the proximal tubule and suggest that the 44-kDa protein may not have a requisite 1:1 stoichiometry with gp330. In addition, immunocytochemical results obtained on rat kidney tissue suggest that the 44-kDa protein is present at the cell surface and in the rough ER: anti-C14p IgG strongly labeled the basal (coated pit) region of proximal tubule microvilli in acetone-fixed cryostat sections, which is in keeping with the fact that when the anti-C14p IgG is injected in vivo it binds to the native HN antigenic complex located in the coated pits at the base of the foot processes (9). By contrast, in aldehyde-fixed tissues labeling was confined to the ER in the proximal tubule and glomerular epithelium, suggesting that there is a significant pool of the protein in the ER. The localization of C14/α2-MRAR in the ER is in keeping with the fact that this protein contains a putative ER retention signal at its C terminus (10, 26).

The initial events of HN are mediated by the binding of circulating antibodies to pathogenic epitopes located in clathrin-coated pits at the base of the foot processes of glomerular

![Fig. 4. Immunocytochemical localization of the 44-kDa, C14p/α2-MRAP protein in rat kidney proximal tubule. (A) Immunofluorescence on acetone-fixed cryostat sections from normal rat kidney incubated with anti-C14p IgG. Apical labeling is seen at the base of the microvilli (MV) of the proximal tubules (arrowheads). (B and C) Similar immunofluorescence preparation of a PLP-fixed kidney section incubated with anti-C14p IgG. Staining is concentrated in the apical cytoplasm. The microvilli (MV) whose position is indicated in the phase-contrast micrograph (C) are unstained. (D) Immunoelectron microscopy revealed that the apical staining seen in the proximal tubule in fixed tissues is due to the presence of the 44-kDa protein in the rough endoplasmic reticulum (ER). L, lumen; N, nucleus; CP, coated pit; MVB, multivesicular body. (A, ×500; B and C, ×400; D, ×18,200.)](image)

![Fig. 5. Immunocytochemical localization of the 44-kDa, C14p/α2-MRAP protein in glomeruli from rat kidney. (A) Immunofluorescence on acetone-fixed cryostat sections resulted in punctate labeling of renal glomeruli (arrowheads) with anti-C14p. (B and C) In fixed tissue, diffuse cytoplasmic staining (arrowhead in C) of glomerular epithelial cells (GEC) was seen under the same incubation conditions. (D) Immunoperoxidase localization of C14p IgG at the electron microscopic level demonstrated the cytoplasmic staining to be due to labeling of the rough ER in glomerular epithelial cells. L, capillary lumen; N, nucleus; US, urinary space; GBM, glomerular basement membrane. (A, ×400; B and C, ×800; D, ×20,800.)](image)
epithelial cells leading to the formation of immune deposits (7, 8). Fig. 6 depicts three models showing how pathogenic epitopes that initiate the onset of HN may be presented at the cell surface. Model I suggests that multiple pathogenic domains exist within and/or between the subunits of the gp330–44-kDa complex. When antibodies eluted from the glomeruli of HN rats were used to screen an expression library, the C14 cDNA sequence was identified and shown to encode a polypeptide containing a pathogenic epitope. An antibody raised against a C14 fusion protein produced immune deposits when injected into rats (9), and when rats were immunized with the C14 fusion protein, they developed active HN. Thus, at least one pathogenic epitope (recently narrowed to 80 amino acids; D.K. and M.G.F., unpublished work) involved in the induction of HN is located within the 44-kDa subunit. However, since antibodies eluted from the glomeruli of HN rats recognize primarily gp330 (5, 6), it is likely that there are multiple epitopes, including one or more present on gp330, responsible for immune complex deposition. These data could also be explained by model II, which suggests that immunologically cross-reactive epitopes are present on gp330 and the C14p/α2-MRAP protein. Though the primary sequence of each subunit may differ, their secondary and tertiary structures may present conformations that are antigenically identical for antibody recognition. Such antigenic cross-reactivity has been demonstrated between human mesangial cell vimentin and the nephritogenic type 12 streptococcal M protein (28). Finally, model III proposes that the presentation of C14p/α2-MRAP is pathogenic when associated with gp330 and nonpathogenic when bound to the α2-macroglobulin receptor. Although α2-MRAP is an abundant protein in liver tissue (29), rats immunized with liver membranes failed to form immune deposits (1). These results suggest that the tertiary structure of the 44-kDa protein is flexible, assuming different conformations dependent on the associating subunit in the heterodimer. Our data additionally suggest that the conformation of the 44-kDa protein is acutely sensitive to fixation. Incubation with C14p IgG results in surface staining when applied to unfixed kidney sections and intracellular (ER) staining in fixed tissue. Such a flexible conformation may permit the 44-kDa protein to adopt different antigenic properties depending on the associated subunit—i.e., gp330 or α2-macroglobulin/LRP receptor.

To determine which model best describes the mechanism of immune complex formation leading to glomerular injury, it will be necessary to obtain the full cDNA sequence for gp330 and study the pathogenicity of protein subdomains. In addition, structural information will be needed to establish whether heterodimer combinations between C14p/α2-MRAP and members of the LDL receptor gene family influence pathogenicity.

This work was supported by National Institutes of Health Grant DK17724 (to M.G.F.) and Austrian Fonds zur Forderung der Wissenschaftlichen Forschung, Contract p7742 MED (to D.K.). R.A.O. was supported by Public Health Service Award NS07078-13.