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Detection of Two Forms of GP330

Their Role in Heymann Nephritis

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Heymann nephritis is characterized by glomerular immune deposits that contain a glycoprotein called gp330. The deposits are believed to result from shedding of immune complexes formed on podocytes. Complexes are also shed from proximal tubule cells, when antibodies combine with gp330 on the cell surface. We performed the present study to investigate what portion of the gp330 molecule is shed, using a rabbit antiserum against a peptide deduced to be in the cytoplasmic domain of gp330, as well as a rabbit antiserum and two monoclonal antibodies that recognize extracellular epitopes of gp330. The anti-cytoplasmic peptide antiserum precipitated from Fx1A (a crude renal cortical membrane preparation), a protein with a mass of about 440 kd that was reactive with two monoclonal anti-gp330 antibodies. (In our experiments, the protein called gp330 generally has a mass estimated to be about 440 kd.) The anti-cytoplasmic peptide antiserum also reacted with a truncated gp330 protein produced in transfected COS cells. Immunohistochemical studies showed that all the antibodies recognized the same group of epithelial cells. However, as seen in immunoultrastructural studies of proximal tubules, the anti-cytoplasmic peptide antiserum reacted only with components at the base of microvilli, whereas the anti-gp330 ectodomain antibodies identified material not only at the base, but over the surface of microvilli as well. In rats with Heymann nephritis, glomerular deposits and material shed into tubule lumens reacted with antibodies against extracellular epitopes of gp330, but not with the anti-cytoplasmic peptide antiserum. We propose that there are two forms of gp330 on the cell surface of proximal renal tubules. One form is restricted to coated pit regions at the base of microvilli and has a cytoplasmic domain containing a sequence deduced from a partial complementary DNA encoding gp330. The other form is present over microvilli (and possibly at the base of microvilli as well) and lacks the cytoplasmic domain deduced from the complementary DNA. The complexes that are shed in Heymann nephritis contain either a portion of gp330 cleaved from the full-length molecule or a form of gp330 that lacks the cytoplasmic domain. (Am J Pathol 1993, 143:598–611)

Heymann nephritis, a model in rats of human membranous glomerulonephritis, is induced by immunization with renal cortical preparations in adjuvants (active Heymann nephritis) or by administration to normal rats of heterologous antibodies against renal antigens (passive Heymann nephritis),[1] reviewed in ref. 2. The lesions are characterized by immune deposits on the outer side of the glomerular basement membrane. The best-characterized antigen in Heymann nephritis is a large glycoprotein, called gp330,[3] which is found on podocytes (in rats)[4] and on apical domains of certain other epithelial cells, notably proximal renal tubule cells, Type II pneumocytes, and epididymal cells.[5,6] There are conflicting observations concerning the precise location of gp330 on renal tubule cells. Some investigators have reported that

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gp330 is restricted to coated pit regions,[7,8] whereas we[9] and others[10] have found gp330 also on the surface of microvilli in some segments of proximal tubules.

The function of gp330 is unknown, but amino acid sequences deduced from partial complementary DNA[11] reveal homology with the low-density lipoprotein (LDL) receptor[12] and the LDL receptor-related protein (LRP), which is also called the α2 macroglobulin receptor (α2MR).[13–15] This finding, together with its location in coated pits, indicates that gp330 may be a receptor. Evidence has been reported indicating that gp330 is a receptor for plasminogen[16] and for extracellular matrix components.[17] Nevertheless, because in most locations gp330 is not normally exposed to these molecules, it is likely that other ligands remain to be identified.

Kerjaschki et al have studied the formation of glomerular immune deposits following injection of rabbit anti-rat gp330 antibodies into normal rats.[18] Within 15 minutes, rabbit IgG and rat gp330 were seen in deposits in the GBM, especially adjacent to coated pits. They postulated that deposits result from shedding of immune complexes formed on the podocyte surface. Support for this hypothesis was provided by experiments using cultured glomerular epithelial cells in which antibodies against surface antigens resulted in patching and capping, followed by internalization and/or shedding of complexes.[19] Shedding of gp330 in immune complexes also seems to occur from proximal tubule cells in Heymann nephritis, as a consequence of increased glomerular permeability, which allows passage of plasma proteins into tubules and the combination of antibodies with brush border gp330.[20,21]

The components of gp330 in the immune deposits of Heymann nephritis have not been determined. In particular, it is not known how much of the protein is shed from the cell surface. Recently acquired knowledge of the primary structure of gp330 makes it possible to investigate this question. Based on amino acid sequences derived from partial cDNA clones, gp330 is predicted to have a very large extracellular domain, a 29-amino-acid transmembrane sequence and a 188-amino-acid cytoplasmic domain at the carboxy (COOH)-terminus.[11] A protein with a different sequence was described by Pietromonaco et al as part of gp330.[22] However, subsequent studies showed it to be a separate protein,[23] with an estimated mass of 39 to 44 kd, which complexes with gp330[24,25] and the LRP/α2MR.[26]

In the present study, we used a rabbit antiserum against a synthetic peptide deduced to be in the cytoplasmic domain of gp330, as well as antibodies reactive with epitopes on the extracellular domain, to investigate the composition of gp330 in immune complexes in Heymann nephritis.

Materials and Methods

Antibodies

A monoclonal anti-gp330 antibody, 14C1,[9] and a rabbit anti-gp330 antiserum were prepared as described previously.[27] An additional monoclonal antibody (1H2) was obtained by immunizing mice with immunoaffinity-purified gp330; enzyme-linked immunosorbent assay and Western blot analysis showed reactivity of 1H2 with purified gp330 (Sy and Yahya, unpublished). 1H2 has also been shown to react with gp330 affinity-purified on a column containing the 39 to 44-kd receptor-associated protein.[25] For immunoblotting and immunohistochemical studies, the monoclonal antibodies were purified from hybridomas supernatants by protein A Sepharose affinity chromatography. Both 14C1 and 1H2 are IgG1 antibodies.

Antiserum against an 18-amino-acid peptide, deduced from a previously cloned partial cDNA[11] to be in the cytoplasmic domain of gp330, was obtained as follows. The synthetic peptide C-M-E-V-G-K-Q-P-V-I-F-E-N-P-M-Y-A-A was produced with an Applied Biosystems solid phase peptide synthesizer. For use as an immunogen, the peptide was coupled to keyhole limpet hemocyanin (KLH), using the linking agent, m-maleimidobenzoyl-N-hydroxy succinamide ester. Ten mg of KLH, 6 mg of m-maleimidobenzoyl-N-hydroxy succinamide ester (dissolved in 100 µl of dimethyl formamide/tetrahydrofuran 50/50), and 6 mg of the synthetic peptide were stirred at room temperature in 7 ml of 0.1 mol/L potassium phosphate buffer (pH 7.3) for 16 hours.[28]

A rabbit was injected intradermally with 1 mg of the KLH peptide in complete Freund's adjuvant on day 0 and with KLH peptide in incomplete Freund's adjuvant at seven intervals between days 29 and 267. Serum samples were obtained at intervals between days 29 and 267 and were tested for reactivity with the peptide (coupled to bovine serum albumin) by enzyme-linked immunosorbent assay. Bovine serum albumin peptide was incubated in wells of microtiter plates in buffer at a concentration of 1 mg/ml. Wells were washed three times with borate-buffered saline, pH 8.3, and then blocked with 2% nonfat powdered milk in borate-buffered

saline. The rabbit antiserum or preimmune serum was added at dilutions of 1:200 or 1:1000 in 1% milk, and after 60 minutes the wells were washed three times with borate-buffered saline and then incubated with a 1:500 dilution of goat anti-rabbit IgG coupled with alkaline phosphatase. Analysis was performed with nitro blue tetrazolium/5-bromo-4-chloro-3-indol phosphate as substrate and reaction product measured at 405 nm. Six weeks after the start of immunization, antibodies reactive with the synthetic peptide were detected by enzyme-linked immunosorbent assay. The specificity of the antiserum was further assessed by immunofluorescence studies of normal rat tissue and by Western blot and immunoprecipitation studies with Fx1A, as described below.

Active Heymann Nephritis

Three female Lewis rats (weighing 160 to 200 g) were immunized by intradermal injection of 4 mg of Fx1A in 0.2 ml of complete Freund's adjuvant together with a separate injection of 0.1 μg of B. pertussis, as previously described.[29] Four weeks later, 1 mg of Fx1A in incomplete Freund's adjuvant was administered intradermally. Proteinuria was monitored by Albustix (Miles Inc., Elkhart, IN). The animals were sacrificed when they developed 3+/4+ proteinuria, at about 7 weeks after the first injection. Nonimmunized animals served as controls.

Passive Heymann Nephritis

Four normal Lewis rats were unilaterally nephrectomized under anesthesia, and 3 days later two rats were injected intravenously with 2 ml of rabbit antiserum against gp330 and two rats with the rabbit anti-cytoplasmic peptide antiserum. The rats were sacrificed 3 days later.

Tissue Processing

For some immunofluorescence studies, animals were anesthetized with pentobarbital (Inactin Byk Gulden, Konstanz, FRG) and then perfused for 10 minutes with a paraformaldehyde lysine-periodate (PLP) solution[30] administered via the left ventricle. Small pieces of renal tissue were immersion fixed in PLP at 4 C overnight. The tissue was processed further for either light or electron microscopic immunocytochemical studies, performed as described.[31]

In other experiments, tissue removed from animals that were not perfused was immediately frozen in liquid nitrogen, and sections were cut at 5 μ, dried, fixed in acetone for 5 minutes, and stained by indirect immunofluorescence as previously described.[32] The primary antibodies consisted of the rabbit anti-gp330 antiserum, the rabbit anti-cytoplasmic peptide antiserum, and the two mouse monoclonal anti-gp330-antibodies (14C1 and 1H2). The secondary antibodies used were fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Cappel, Durham, NC) or anti-mouse IgG (Sigma Chemical Co., St. Louis, MO), which had been absorbed with normal rat serum. Sections prepared from kidney, liver, adrenal, lung, and epididymis were studied.

Immunoelectron Microscopy

For detection of antigenic sites at the ultrastructural level, immunogold labeling of ultrathin frozen sections was used, prepared by the method of Tokuyasu.[33] Briefly, pieces of PLP-fixed kidney cortex were cryoprotected in 2.3 mol/L sucrose for at least 1 hour, frozen in liquid nitrogen, and sectioned using a Reichert FC4D ultracryomicrotome. Ultrathin sections were picked up on nickel grids and stored at 4 C on phosphate-buffered saline (PBS), pH 7.4, containing 1% gelatine until use. Sections were incubated for 1 hour at room temperature, or at 4 C overnight in a dilution of the rabbit anti-gp330 antiserum (1:200) or the anti-cytoplasmic peptide antiserum (1:100). Sections were washed three times for 5 minutes in PBS, and then incubated for 1 hour on a drop of protein A-gold prepared with 15-nm gold particles as previously described.[34] For double labeling, sections were incubated in the following sequence: 1 hour in primary mouse monoclonal anti-gp330 antibody (14C1) (20 μg/ml); 1 hour in goat anti-mouse IgG coupled to 10-nm gold particles (Janssen, Piscataway, NJ); 1 hour in primary anti-cytoplasmic peptide antiserum; and finally 1 hour in 15-nm protein A-gold, which under the conditions of incubation binds only to the rabbit anti-cytoplasmic peptide antiserum. Sections were washed in PBS, fixed for 10 minutes in 1% glutaraldehyde, and washed again in distilled water. They were stained for 5 minutes in 2% aqueous uranyl acetate and embedded in methyl cellulose before examination in a Philips CM10 electron microscope.

Western Blot Analysis

This was performed with Fx1A prepared from kidneys perfused *in vivo* with phenylmethyl sulfonyl fluoride, and ethylenediaminetetraacetic acid. The

Fx1A was separated by nonreducing, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 4 to 12% gradient gel and transferred to nitrocellulose. The monoclonal anti-gp330 antibodies, the anti-gp330 antiserum, and the anti-cytoplasmic peptide antiserum all reacted with material in the region of 440 kd. (It is now recognized that the protein called gp330 often seems to have a higher molecular mass, depending on the conditions of the gel.[24]) In addition, other bands were seen, as described in results.

Immunoprecipitation of Proteins in Fx1A with the Anti-Cytoplasmic Peptide Antiserum

One hundred μl of normal rabbit serum or rabbit anti-cytoplasmic peptide antiserum were added to 100 μl of Sepharose protein A beads along with 1 ml of PBS. After 1 hour of incubation at 4 C on a tube rotator, the beads were pelleted and the supernatant discarded. Fx1A, prepared by solubilization in 1% deoxycholate,[35] was precleared by incubation with Sepharose protein A beads for 1 hour at 4 C on a tube rotator followed by centrifugation for 5 minutes at 2,000 rotations/minute. One mg of precleared, solubilized Fx1A was then added to each of the tubes containing the beads that had been incubated with the normal rabbit serum or the anti-cytoplasmic peptide antiserum. After 1 hour of incubation, the beads were washed six times with 50 mm Tris (pH 8.6), containing 1% deoxycholate and 1% Triton (X-100), and twice in PBS. The beads were then suspended in nonreducing SDS sample buffer, boiled for five minutes, and after a final centrifugation, the supernatant was separated on SDS gels and transferred to nitrocellulose paper. Strips of the paper were blocked with 1% nonfat dried milk and then incubated with the monoclonal anti-gp330 antibodies, (14C1 and 1H2) and with an irrelevant isotype-matched monoclonal antibody (1E8) against a human granulocyte serine protease.[36] Staining was produced by use of an avidin biotin peroxidase secondary antibody system (Vectastain, Vector Laboratories, Burlingame, CA).

Expression of a Truncated gp330 Protein in COS Cells

The reactivity of the anti-gp330 antibodies was also assessed by use of COS 7 cells[37] that were transfected with a cDNA that encodes part of gp330, including 423 amino acids of the extracellular domain, 29 amino acids of the transmembrane domain, and 188 amino acids representing the complete cytoplasmic domain.[11] The cDNA was ligated into the CDM8 expression vector with the signal sequence for the complement Type 1 receptor. This construct was used to target the truncated gp330 protein into the lumen of the endoplasmic reticulum and then to the cell surface. As controls, COS cells were transfected with cDNA encoding the human LDL receptor American Type Culture Collection or with the CDM8 vector (Invitrogen, San Diego, CA) alone.

COS cells were incubated with 10 μg of the plasmid construct in the presence of DEAE-Dextran and 10% Nuserum (Collaborative Research).[37] Incubation at 37 C for 6 hours was followed by a 2-minute shock of 10% dimethylsulfoxide in PBS. Cells were placed in Dulbecco's minimum essential medium and 10% fetal bovine serum supplemented with glutamine and then trypsin treated and replated after 24 hours. For Western blot analysis, cells were lysed in detergent buffer (1% deoxycholate in PBS) and separated by 10% SDS-PAGE in nonreducing conditions (using Protogel from National Diagnostics). The separated proteins were transferred to nitrocellulose and incubated with the appropriate primary antibodies. For immunofluorescence staining, cells were fixed with 3% paraformaldehyde in PBS at 4 C for 4 hours. Some cells were then incubated with the rabbit anti-gp330 antiserum or the anti-cytoplasmic peptide antiserum for 1 hour at room temperature, washed, and then incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG for 1 hour. Other cells were permeabilized with 0.1% Triton X-100 in PBS for 5 minutes, washed three times, and then incubated with the rabbit anti-cytoplasmic peptide antiserum, followed by the conjugated secondary antiserum. Cells were examined and imaged using a Biorad 600 laser confocal microscope.

Results

Immunoprecipitation of gp330 from Fx1A by the Anti-Cytoplasmic Antiserum

Proteins immunoprecipitated from Fx1A-DOC by the anti-cytoplasmic peptide antiserum coupled to Sepharose beads were separated by SDS-PAGE and transferred to nitrocellulose paper. As shown in Figure 1, two monoclonal anti-gp330 antibodies reacted with an identical band, which had a mass

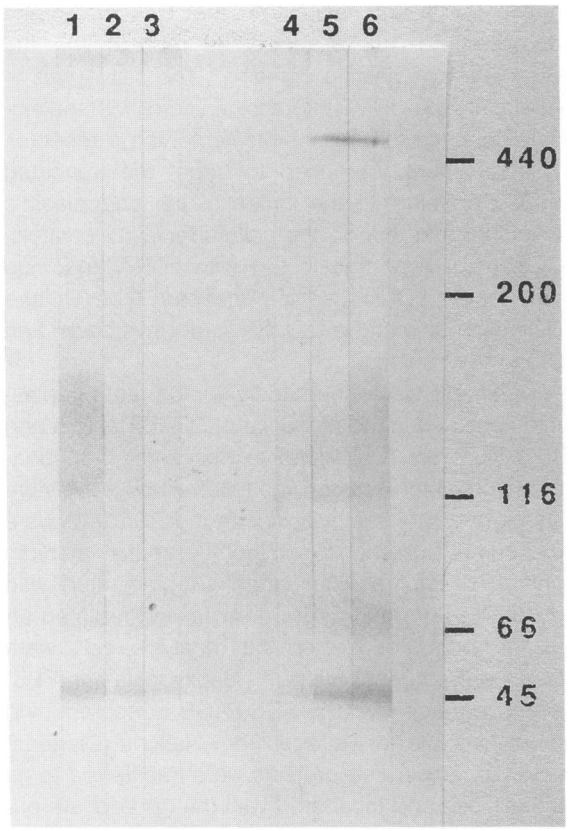

Figure 1. *Material in soluble Fx1A that bound to the rabbit anti-cytoplasmic peptide antiserum on protein A beads was separated by SDS-PAGE and transferred to nitrocellulose paper* (lanes 4 *to* 6). *Normal rabbit serum on protein A beads was used as a control* (lanes 1 *to* 3). *The monoclonal anti-gp330 antibodies (1H2 and 14C1,* lanes 5 *and* 6) *resulted in staining of a band slightly above 440 kd, whereas the irrelevant monoclonal antibody (1E8,* lane 4) *did not. No staining of a 440-kd band was seen when normal rabbit serum was used. The markers on the right are based upon fibronectin (440) and the Bio-Rad high molecular weight markers.*

slightly greater than 440 kd. An irrelevant monoclonal antibody (1E8) did not react with the immune precipitate. Normal rabbit serum did not precipitate this protein. The results show that the anti-cytoplasmic peptide antiserum recognizes gp330.

Western Blot Analysis

On Western blots, using Fx1A, the monoclonal anti-gp330 antibodies, and the anti-cytoplasmic peptide antiserum, all reacted with material in the region of 440 kd; however, the staining with the anti-cytoplasmic peptide antiserum was weaker and more restricted than that produced by the other antibodies. The anti-cytoplasmic peptide antiserum stained material only in the upper part of the 440-kd region, whereas the monoclonal anti-gp330 antibodies stained a broader region, which included material in the upper part (Figure 2). The anti-cytoplasmic peptide antiserum also produced faint staining in lower molecular weight regions, particularly in the regions of 110 kd and 80 kd. The proteins in these lower molecular weight regions were not stained by the monoclonal anti-gp330 antibodies.

COS Cells Transfected with a gp330 cDNA

Western blot analysis of extracts of COS cells transfected with cDNA encoding part of gp330 (with a predicted mass of 77 kd) showed two bands at 80 and 74 kd, recognized both by the rabbit anti-gp330 antiserum and the anti-cytoplasmic peptide

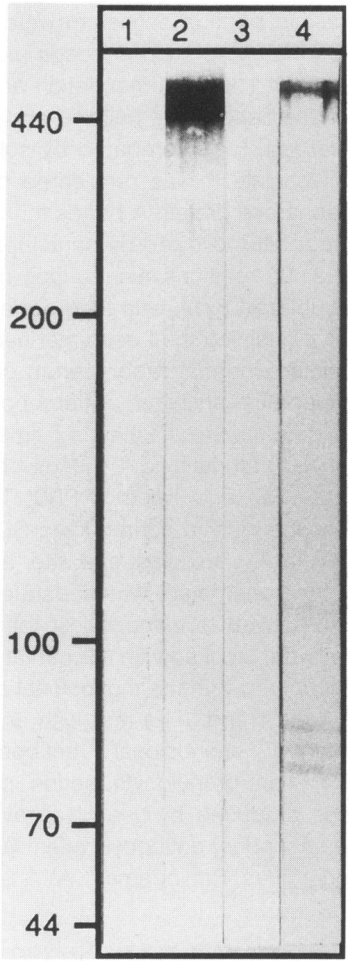

Figure 2. *Western blot analysis of Fx1A separated by 4 to 12% gradient nonreducing SDS-PAGE and transferred to nitrocellulose.* Lane 1: *control mouse monoclonal antibody;* lane 2: *anti-gp330 monoclonal antibody (14C1);* lane 3: *normal rabbit serum; and* lane 4: *anti-cytoplasmic peptide antiserum. The markers on the left are based upon fibronectin (440) and the Rainbow high molecular weight markers from Amersham.*

antiserum (Figure 3). The antibodies did not react with similar bands in extracts of control COS cells. Treatment of extracts of transfected COS cells with N-glycanase and O-glycanase (Genzyme, Cambridge, MA) resulted in the elimination of the upper band (data not shown), indicating that this material differed from the lower because of glycosylation.

Immunofluorescence microscopy was performed with transfected and control COS cells (Figure 4). The rabbit anti-gp330 antiserum produced punctate staining of PLP-fixed cells, but not of control nontransfected cells. The anti-cytoplasmic peptide antiserum produced staining of PLP-fixed transfected cells; however, there was background staining of control cells. Following permeabilization with 0.1% Triton X-100, the intensity of staining of transfected COS cells by the anti-cytoplasmic antiserum was markedly increased, whereas the staining of control cells was unchanged.

Immunofluorescence Studies in Normal Rats

Immunofluorescence studies of several organs showed that the anti-cytoplasmic peptide antiserum, the anti-gp330 antiserum, and the monoclonal antibodies reacted with a group of epithelial cells known to express gp330, namely, renal proximal tubule cells, Type II pneumocytes, and epididymal cells. None of the antibodies reacted with hepatic cells or with adrenal cortical cells, which express the homologous proteins, LPR/α2MR[38] and LDL receptor.[39]

Although all cells that stained with the anti-gp330 antibodies also stained with the anti-cytoplasmic peptide antiserum, there were slight dissimilarities in the staining patterns in certain cells. The differences were clearly seen in renal proximal tubules, especially in 1-μ sections of PLP-fixed tissue (Figure 5). The polyclonal and monoclonal anti-gp330 antibodies resulted in a broad zone of staining, which seemed to be present not only at the base of microvilli, but over the entire brush border microvilli as well (Figure 5A). This microvillar staining varied in intensity in different segments of the proximal tubule. In contrast, the reactivity of the anti-cytoplasmic peptide antiserum was restricted to a narrow zone, which seemed to be at the base of the intermicrovillar region (the intermicrovillar microdomain, Figure 5B).

Immunofluorescence microscopy also revealed differences in the reactivity of the antibodies with the podocytes of normal glomeruli. The anti-cytoplasmic peptide serum produced only

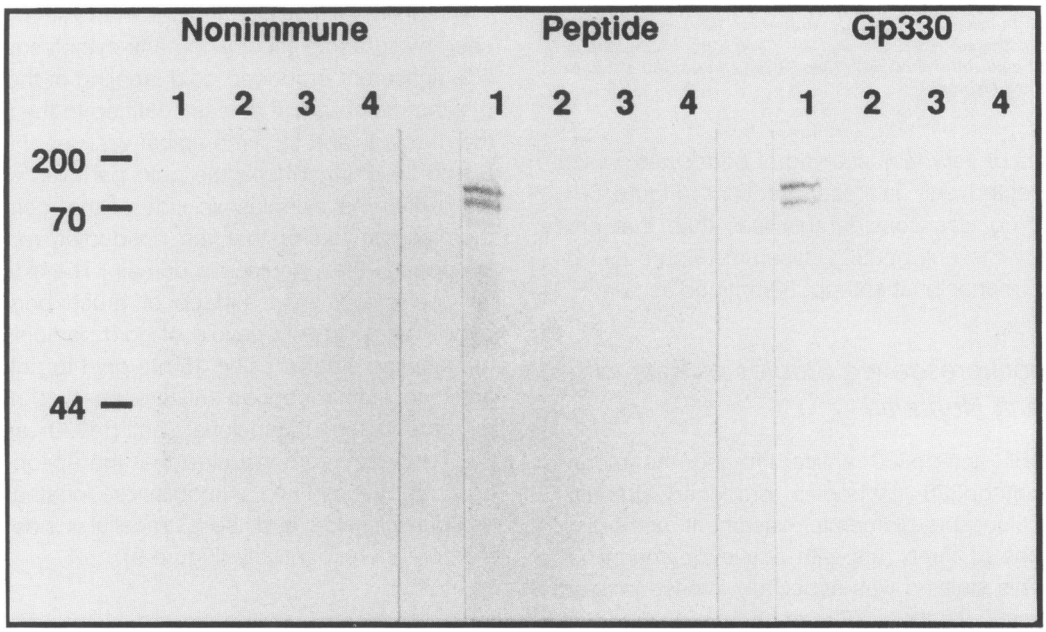

Figure 3. *Western blot analysis of extracts of COS cells that had been transfected with partial gp330 cDNA* (lane 1), *with LDL receptor cDNA* (lane 2), *CDM8 vector* (lane 3), *or no DNA* (lane 4). *Extracts were prepared 48 hours after transfection and separated on nonreducing 10% SDS-PAGE. The blots were stained using normal rabbit serum* (nonimmune), *anti-cytoplasmic peptide serum* (peptide), *or anti-gp330 serum* (*gp330*). *The markers on the left are based upon high molecular weight protein standards (Gibco Bethesda Research Laboratories, Bethesda, MD). The anti-cytoplasmic and the anti-gp330 antisera both stained bands in the extracts of COS cells transfected with partial gp330 cDNA, but not in the control extracts.*

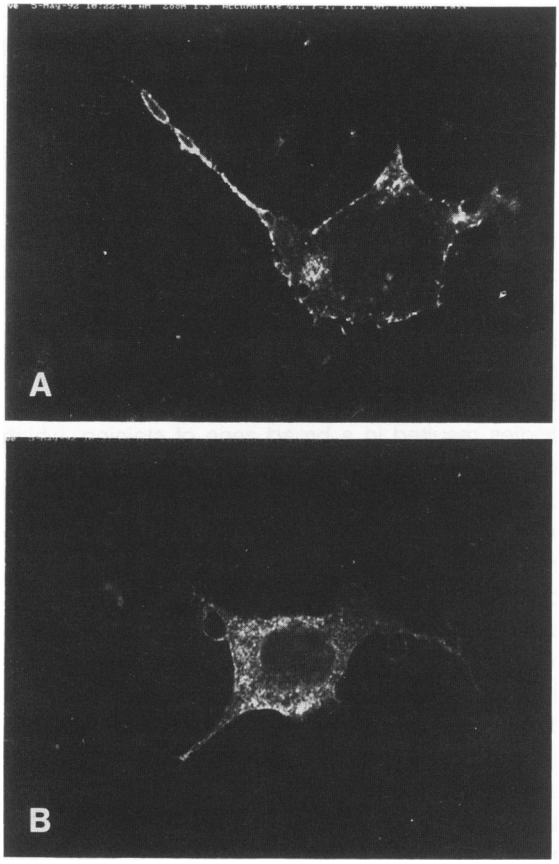

Figure 4. *Confocal immunofluorescence images of COS cells transfected with the partial gp330 cDNA. The cells were incubated with rabbit anti-gp330 antiserum* (**A** *using PLP-fixed nonpermeabilized cells*) *or anti-cytoplasmic peptide antiserum* (**B** *using PLP-fixed then permeabilized cells*), *followed by fluorescein isothiocyanate-conjugated goat anti-rabbit IgG. There is punctate staining that extends into cell processes.*

equivocal or very faint staining of podocytes, which was detected only in frozen sections (Figure 6B); the staining was considerably less than that produced by the anti-gp330 antiserum (Figure 6A) or even the monoclonal anti-gp330 antibodies.

Immunofluorescence Studies of Rats with Heymann Nephritis

The rabbit anti-gp330 antiserum and the monoclonal anti-gp330 antibodies produced granular staining along the glomerular basement membrane in sections of three rats with active Heymann nephritis. This staining was especially intense in fresh frozen tissue sections (Figure 6C), but was also seen in PLP-fixed tissue (Figure 7A). In addition, there was staining of luminal debris, which reflects shedding of gp330 containing immune complexes into the tubule lumen.[21] Compared with normal kidneys, the brush border staining for gp330 was diminished or focally lost. However, some residual staining was seen at the base of the microvilli, even in areas where there was an absence of microvillar staining. In contrast, the rabbit anti-cytoplasmic peptide antiserum failed to stain glomerular deposits or debris in the lumen of proximal tubules (Figures 6D and 7B). However, the regions at the base of the microvilli of proximal tubules were intensely stained (Figure 7B).

In passive transfer experiments, the kidneys of the two rats that received rabbit anti-gp330 antiserum 3 days earlier showed bright, granular staining for rabbit IgG of glomerular deposits and brush border regions. In contrast, there was no staining for rabbit IgG in the two rats that received the anti-cytoplasmic peptide antiserum.

Immunogold Studies

Further information about the distribution of gp330 in normal renal tubules was obtained by immunogold electronmicroscopic studies. The rabbit anti-gp330 antiserum produced gold labeling of brush border microvilli, the base of the microvilli, in the region of coated pits, and some apical vesicles (Figure 8, A and C). As seen in perpendicular sections through apical membranes, the reactive sites were clearly extracellular.

In confirmation of the findings in proximal tubules seen by light microscopy, the anti-cytoplasmic peptide antiserum produced gold labeling of the apical plasma membrane that was confined to the base of the microvilli and to some apical vesicles (Figure 8, B and D). Furthermore, the gold particles were restricted to the cytoplasmic side of the membrane, thus demonstrating that the antibodies recognize epitopes in the cytoplasmic domain. The extracellular and cytoplasmic surface of brush border microvilli were virtually devoid of gold particles. Double labeling studies using 15-nm gold to detect the anti-cytoplasmic peptide antiserum and 10-nm gold to detect the monoclonal anti-gp330 antibody (14C1) clearly demonstrated that the epitopes recognized by these two antibodies are located on the cytoplasmic side and the extracellular side of the membrane, respectively (Figure 9).

Discussion

We designed the present study to investigate what part of the gp330 molecule is present in immune complexes shed from the plasma membrane of

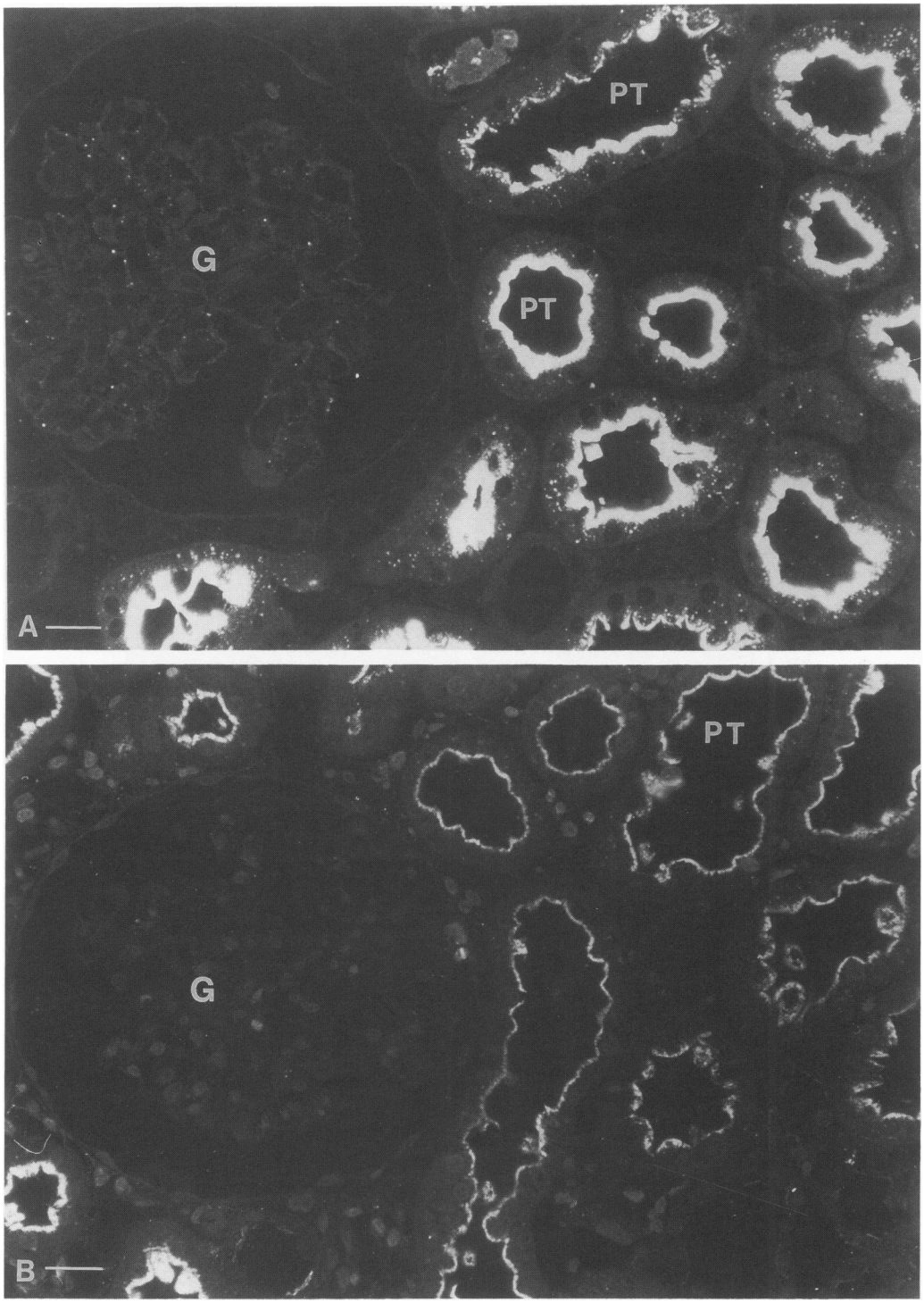

Figure 5. *Immunofluorescence micrograph of PLP-fixed normal rat kidney cortex. The anti-gp330 antiserum* **(A)** *produces a broader band of staining of the proximal tubule (PT)* brush border region than the anti-cytoplasmic peptide antiserum **(B)**. *The brush border staining pattern produced by the anti-gp330 antiserum varies among tubules. The glomerulus (G) shows faint staining with the anti-gp330 antiserum, but is not stained with the anti-cytoplasmic peptide antiserum serum. (Scale bar = 20 μ)*

podocytes or proximal tubule cells following combination with antibodies in Heymann nephritis. For this purpose, we used monoclonal and polyclonal antibodies that recognize extracellular epitopes of gp330 and a rabbit antiserum produced by immunization with a peptide sequence predicted from a partial cDNA encoding gp330 to be in the cytoplasmic domain.[11] We found that antibodies directed

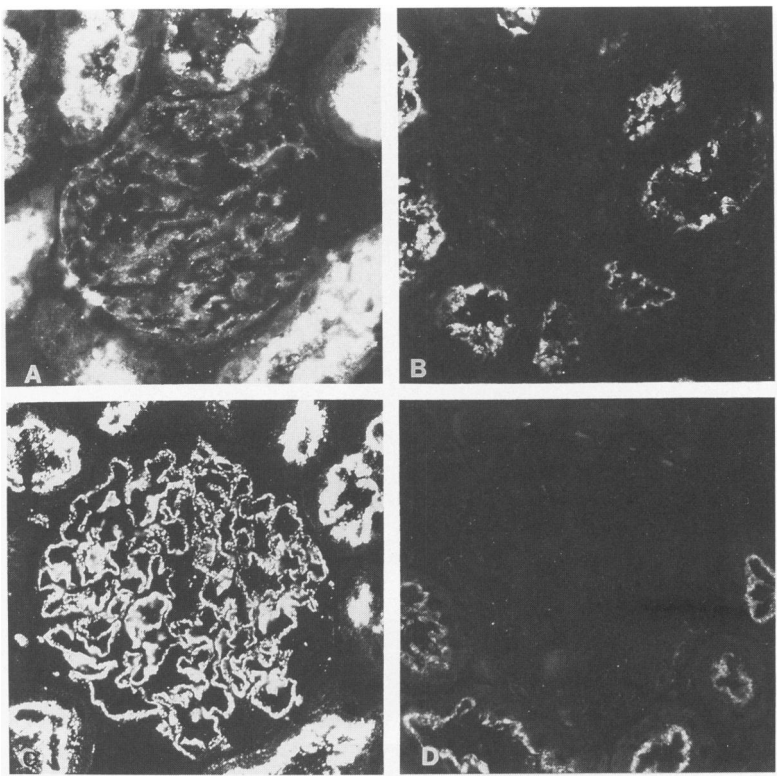

Figure 6. *Immunofluorescence micrographs of sections prepared from fresh-frozen, acetone-fixed kidney of a normal rat* (A *and* B) *and a rat with Heymann nephritis* (C *and* D). *The anti-gp330 antiserum produces conspicuous punctate staining of the normal glomerulus* (A) *and intense staining of deposits in a rat with Heymann nephritis* (C). *The anti-cytoplasmic peptide antiserum produces only very faint staining of the normal glomerulus and fails to stain the immune deposits* (B *and* D).

against extracellular epitopes reacted intensely with material in the glomerular deposits and debris in tubular lumens, whereas the anti-cytoplasmic peptide antiserum did not react. Furthermore, administration of the rabbit anti-gp330 antiserum produced passive Heymann nephritis and the anti-cytoplasmic antiserum failed to do so. These findings indicate that the gp330 in immune deposits is derived mainly from the extracellular domain, whereas part or all of the cytoplasmic domain is not shed into deposits.

We initially interpreted these results as indicating that the full-length gp330 molecule is cleaved somewhere in the extracellular domain during the formation of immune deposits. We had assumed that there is only one type of gp330 on the cell surface, consisting of a continuous polypeptide chain, with the predicted cytoplasmic and transmembrane domains and a very large ectodomain. However, certain immunohistochemical findings in the brush border of proximal tubules led us to hypothesize that there are two separate forms of gp330 and that only one of these is found in the immune deposits. One form of gp330, which is identified by our anti-cytoplasmic peptide antiserum, is restricted to clathrin-coated pit regions and apical vesicles of proximal tubules. The second form, which is reactive with antibodies against extracellular epitopes, but not with the cytoplasmic sequence, is found over the microvilli; it could not be determined whether this type is present in coated pit regions as well.

The validity of the hypothesis that there are two forms of gp330 in the kidney depends on the evidence that the anti-cytoplasmic peptide antiserum reacts with a portion of gp330 rather than with a separate protein. The evidence for this conclusion is as follows. First, the antiserum was raised against a peptide whose amino acid sequence was deduced from partial cDNAs encoding gp330.[11] The gene bank did not reveal an identical sequence in other proteins. Second, and most important, the rabbit anti-cytoplasmic peptide antiserum precipitated from Fx1A a protein of about 440 kd that was specifically recognized by two monoclonal anti-gp330 antibodies (under the conditions we use, gp330 has a mass of about 440 kd). Third, the anti-cytoplasmic peptide antiserum stained material in Fx1A with a mass of approximately 440 kd. Fourth, immunofluorescence studies on normal rat tissues

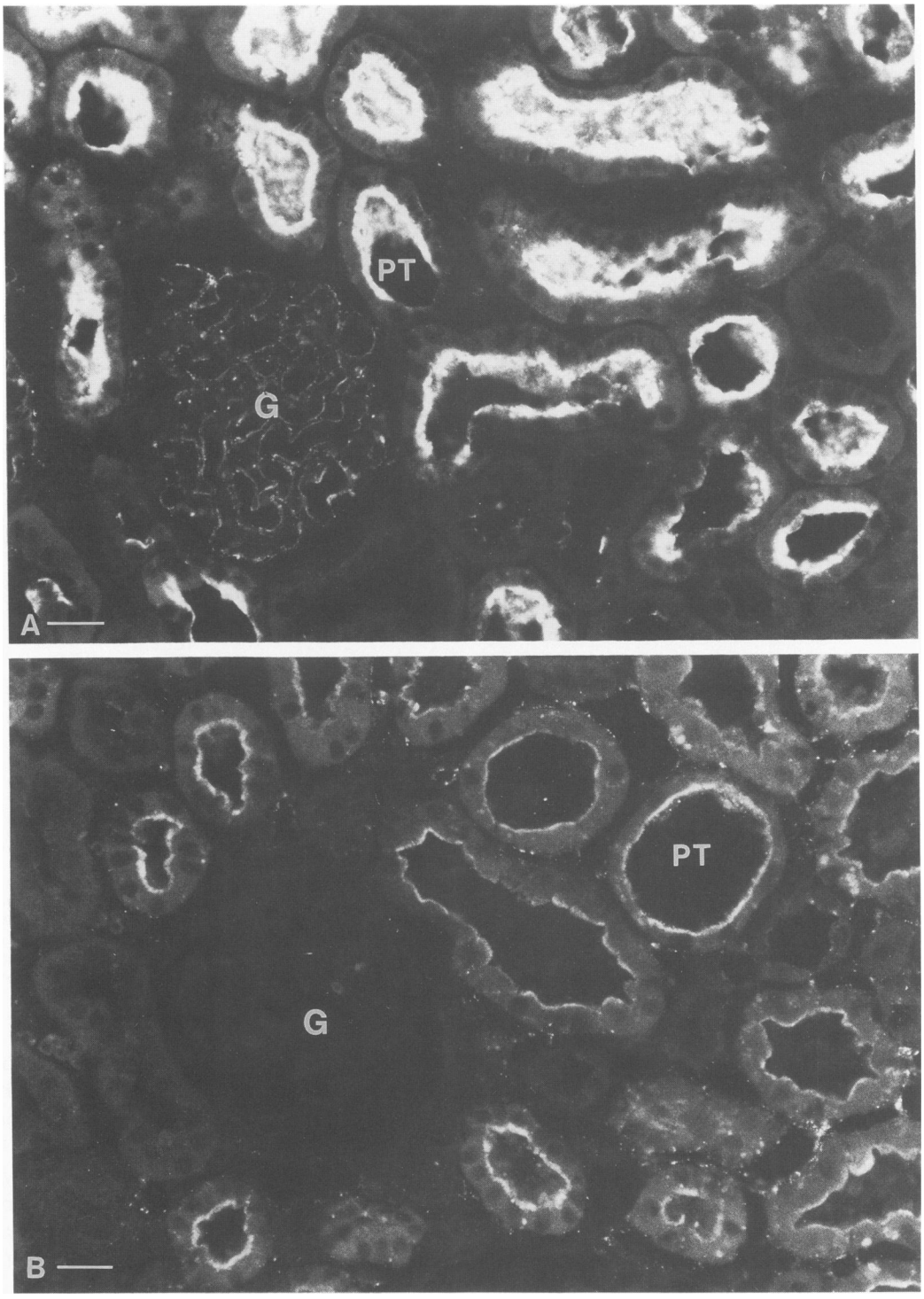

Figure 7. *Immunofluorescence micrographs of PLP-fixed kidney from a rat with Heymann nephritis. The anti-gp330 serum* **(A)** *produces granular staining of deposits in glomerulus* **(G)**, *which is less intense than in frozen sections (Figure 6C). In addition, there is staining of material in the lumen of some proximal tubules (PT) and focal loss of brush border staining. The anti-cytoplasmic peptide antiserum* **(B)** *fails to stain the glomerulus* **(G)** *or luminal debris in proximal tubules (PT) but does stain the base of the brush border, as was seen in normal rats. (Compare with Figure 5B.)*

showed that the anti-cytoplasmic peptide antiserum reacted with cells known to express gp330, namely, renal epithelial cells, epididymal epithelial cells, and Type II pneumocytes, and failed to react with adenal cortical or hepatic cells, which express the homologous proteins, LDL receptor and LRP/α2MR.

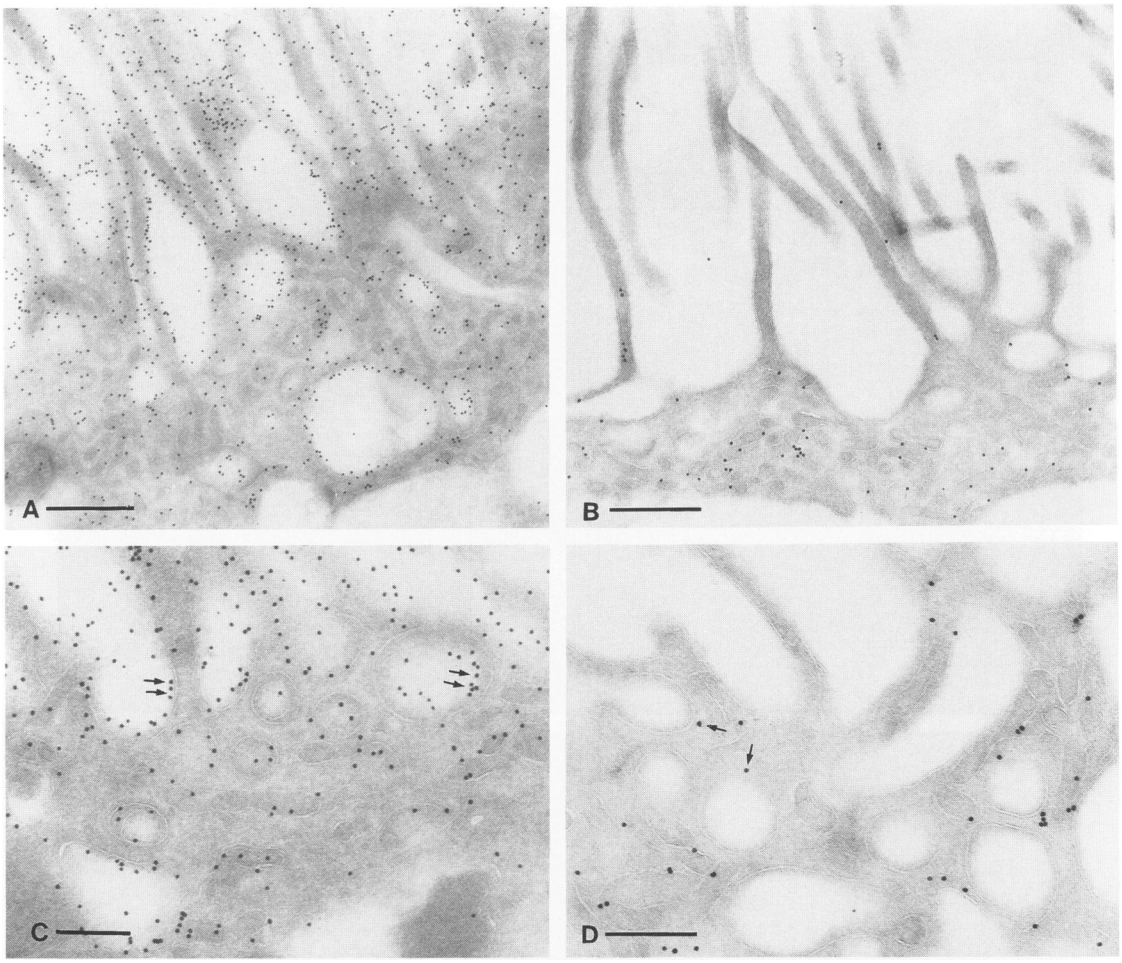

Figure 8. *Ultrathin frozen sections of proximal convoluted tubule epithelial cells from a normal rat kidney incubated with anti-gp 330 antiserum* (**A** *and* **C**) *or anti-cytoplasmic peptide antiserum* (**B** *and* **D**), *followed by 15-nm protein A-gold. The sites recognized by the anti-gp 330 antiserum are located over the surface of the brush border microvilli and in the intermicrovillar region, as well as in apical invaginations and vesicles. At higher magnification* (**C**), *the extracellular location of the reactive sites is clearly detectable in regions where the membrane is sectioned in a perpendicular fashion (arrows). Immunogold labeling with anti-cytoplasmic peptide antiserum is confined to the base of the microvilli, and to some apical vesicles* (**B**); *the surface of brush border microvilli is devoid of staining. The distinct cytoplasmic location of the antigenic sites in perpendicular sections of the apical membrane is shown in* **D** (*arrows*). *Scale bars = 0.5 μ.*

Fifth, the rabbit antiserum against gp330 and the anti-cytoplasmic peptide antiserum both reacted with proteins of the same mass in COS cells transfected with a partial cDNA encoding the cytoplasmic domain, the transmembrane domain, and a portion of the extracellular domain of the gp330 molecule.

Nevertheless, the possibility must still be considered that the protein we detected on the surface of microvilli with the anti-gp330 antibodies is not gp330, but rather other proteins with cross-reacting epitopes. In this regard, Kerjaschki et al have concluded that gp330 is confined to the base of microvilli and that the material demonstrated on the surface of microvilli with polyclonal anti-gp330 antibodies is maltase, which shares epitopes with gp330.[7] Another protein to be considered is a glycoprotein called gp280, which has been found on brush border of renal tubules, especially in intermicrovillar regions, and in the yolk sac.[40] We believe, however, that there is compelling evidence for the presence of gp330 on microvilli. In a study by Chatelet et al,[10] four monoclonal anti-gp330 monoclonal antibodies were shown to react with material over the surface of microvilli in some segments of proximal tubules, and we have made similar observations with two monoclonal antibodies (14C1 and 1H2). It is unlikely that all of these monoclonal anti-gp330 antibodies recognize cross-reactive proteins on microvilli. In addition, monoclonal anti-gp330 antibodies failed to stain absorptive intestinal cells,[5] which express abundant maltase. Furthermore, on Western blots of Fx1A, our monoclonal anti-gp330 antibodies stained proteins in the region of 440 kd,

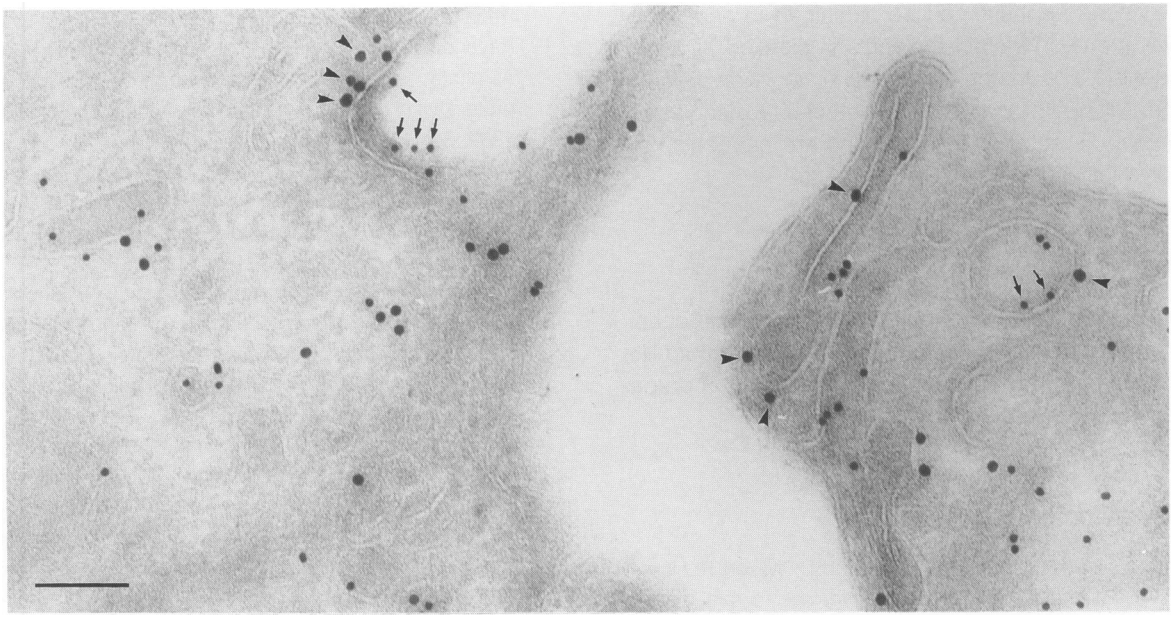

Figure 9. *Double immunogold labeling of the apical region of a proximal convoluted tubule epithelial cell demonstrating localization of extracellular and cytoplasmic domains of gp330. The ultrathin cryosection was incubated first with the monoclonal anti-gp330 antibody (14C1) followed by goat anti-mouse IgG coupled to 10-nm gold particles. The section was then incubated with the anti-cytoplasmic peptide antiserum, which was detected with 15-nm protein A-gold. In the region of coated pits and of apical vesicles, the monoclonal anti-gp 330 antiserum clearly labels the extracellular face of the apical membrane (arrows). In contrast, in the same regions the gold labeling produced by anti-gp 330 cytoplasmic peptide antiserum is restricted to the cytosolic side of the membranes (arrowheads). Scale bar = 0.1 μ.*

but not proteins in lower molecular weight regions that might represent maltase or gp280.

The anti-cytoplasmic peptide antiserum resulted in staining of glomerular podocytes, as seen in frozen tissue sections, but the staining was very faint and markedly less than of proximal tubules. The other anti-gp330 antibodies resulted in more conspicuous staining of podocytes, which was, however, also far less than what was seen in tubules. These findings probably reflect the relatively low level of expression of gp330 by podocytes. In early studies, antigens reactive with antibodies to Fx1A were not detected at all in normal glomeruli by immunofluorescence techniques.[2] It is also possible that the faint staining of glomeruli we observed in the current study was due in part to relatively low titers of the antipeptide antibodies.

The composition and interrelation of the two postulated cell-surface types of gp330 are unknown, but some information is provided by Western blot analysis of Fx1A. The anti-cytoplasmic peptide antiserum was seen to react with material only in the upper region of a broad band around 440 kd that stained with the monoclonal anti-gp330 antibodies and the rabbit anti-gp330 antiserum (Figure 2). These results are consistent with the interpretation that the intact gp330 molecule is represented by protein in the upper region of 440 kd and that the contiguous protein below this lacks a cytoplasmic domain, at least one detectable by our anti-cytoplasmic peptide antiserum. The lower molecular weight materials at about 110 kd and 80 kd recognized by the anti-cytoplasmic peptide antiserum may represent fragments of the parent gp330.

The postulated separation of gp330 into two parts may be analogous to processing of LRP/α2MR, which is split in the trans-Golgi apparatus into an 85-kd portion containing cytoplasmic and transmembrane domains as well as a short extracellular segment and a 515-kd extracellular domain, which is bound noncovalently to the shorter portion.[41] However, we have no evidence that the microvillar type of gp330 is combined with a shorter membrane-spanning segment and do not know how it is attached to the cell membrane.

Although we have speculated that two forms of gp330 arise from intracellular cleavage of a single polypeptide, it is also possible that the different forms are generated by alternative RNA splicing. Still another possibility is that gp330 present over the microvilli possesses a cytoplasmic domain that is conformationally altered in a way that renders it unreactive with the anti-cytoplasmic peptide antiserum.

How does the evidence of two forms of gp330 relate to the formation of immune deposits in Heymann nephritis? The finding that only extracellular epitopes of gp330 were identified in the deposits of

Heymann nephritis can be explained in one of two ways: one, the intact molecule is cleaved at a susceptible site in the ectodomain; or two, only the form of gp330 that lacks a detectable cytoplasmic domain is shed. Further studies are needed to resolve this issue.

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References

1. Heymann W, Hackel D, Harwood S, Wilson S, Hunter J: Production of nephrotic syndrome in rats by Freund's adjuvants and rat kidney suspensions. Proc Soc Exp Biol Med 1959, 100:660–664
2. McCluskey RT: Immunologic aspects of renal disease. Pathology of the Kidney, 4th ed. Edited by Heptinstall, RH. Boston, Little, Brown, and Company, 1992, pp 169–260
3. Kerjaschki D, Farquhar MG: The pathogenic antigen of Heymann nephritis is a membrane glycoprotein of the renal proximal tubule brush border. Proc Natl Acad Sci USA 1982, 79:5557–5561
4. Kerjaschki D, Farquhar MG: Immunological localization of the Heymann nephritis antigen (gp330) in glomerular epithelial cells of normal Lewis rats. J Exp Med 1983, 157:667–686
5. Chatelet F, Brianti E, Ronco P, Roland J, Verroust P: Ultrastructural localization by monoclonal antibodies of brush border antigens expressed by glomeruli. II. Extrarenal distribution. Am J Pathol 1986, 122:512–519
6. Assmann K, Lange W, Tangelder M, Koene R: The organ distribution of gp-330 (Heymann antigen) and gp90 in the mouse and rat. Virchows Arch [A] 1986, 408:541–553
7. Kerjaschki D, Noronha-Blob L, Sacktor B, Farquhar MG: Microdomains of distinctive glycoprotein composition in the kidney proximal tubule brush border. J Cell Biol 1984, 98:1505–1513
8. Biemesderfer D, Dekan G, Aronson P, Farquhar MG: Assembly of distinctive coated pit and microvillar microdomains in the renal brush border. Am J Physiol 1992, 262:F55–F67
9. Bhan A, Schneeberger E, Baird L, Collins A, Kamata K, Bradford D, Erikson M, McCluskey RT: Studies with monoclonal antibodies against brush border antigens in Heymann nephritis. Lab Invest 1985, 53:421–432
10. Chatelet F, Brianti E, Ronco P, Roland J, Verroust P: Ultrastructural localization by monoclonal antibodies of brush border antigens expressed by glomeruli. I. Renal distribution. Am J Pathol 1986, 122:500–511
11. Raychowdhury R, Niles J, McCluskey RT, Smith JA: Autoimmune target in Heymann nephritis is a glycoprotein with homology to the LDL-receptor. Science 1989, 244:1163–1165
12. Yamamoto T, Davis GC, Brown MS, Schneider WJ, Casey LM, Goldstein JL, Russell DW: The human LDL receptor: a cysteine-rich protein with multiple Alu sequences in its mRNA. Cell 1984, 39:27–38
13. Herz J, Hamann U, Rogne S, Myklebost O, Gausepohl H, Stanley KK: Surface location and high affinity for calcium of a 500-kd liver membrane protein closely related to the LDL-receptor suggest a physiological role as lipoprotein receptor. EMBO J 1988, 7:4119–4127
14. Strickland DK, Ascom JD, Williams S, Burgess WH, Migliorini M, Argraves WS: Sequence identity between the alpha 2-macroglobulin receptor and low density lipoprotein receptor-related protein suggests that this molecule is a multifunctional receptor. J Biol Chem 1990, 265:17401–17404
15. Kristensen T, Moestrup SK, Gliemann J, Bendtsen L, Sand O, Sottrup-Jensen L: Evidence that the newly cloned low-density-lipoprotein receptor related protein (LRP) is the alpha 2-macroglobulin receptor. FEBS Lett 1990, 276:151–155
16. Kanalas J, Makker S: Identification of the rat Heymann nephritis autoantigen (gp330) as a receptor site for plasminogen. J Biol Chem 1991, 266:10825–10829
17. Mendrick DL, Chung DC, Rennke HG: Heymann antigen gp330 demonstrates affinity for fibronectin, laminin, and type I collagen and mediates rat proximal tubule epithelial cell adherence to such matrices in vitro. Exp Cell Res 1990, 188:23–35
18. Kerjaschki D, Miettinen A, Farquhar MG: Initial events in the formation of immune deposits in passive Heymann nephritis. J Exp Med 1987, 166:109–128
19. Camussi G, Brentjens J, Nobel B, Kerjaschki D, Malavasi F, Roholt O, Farquhar MG, Andres G: Antibody-induced redistribution of Heymann antigen on the surface of cultured glomerular visceral epithelial cells: possible role in the pathogenesis of Heymann glomerulonephritis. J Immunol 1985, 135:2409–2416
20. Andres G, Brentijens J, Caldwell P, Camussi G, Matsuo S: Formation of immune deposits and disease. Lab Invest 1986, 55:510–520
21. Gutmann E, Niles J, McCluskey RT, Brown D: Loss of antigens associated with the apical endocytotic pathway in proximal tubules from rats with Heymann nephritis. Am J Pathol 1991, 138:1243–1255
22. Pietromonaco S, Kerjaschki D, Binder S, Ullrich R, Farquhar MG: Molecular cloning of a cDNA encoding a major pathogenic domain of the Heymann nephritis antigen gp330. Proc Natl Acad Sci USA 1990, 87: 1811–1815
23. Strickland DK, Ashcom JD, Williams S, Battey F, Behre E, McTigue K, Battey JF, Argraves WS: Primary struc-

ture of alpha-2 macroglobulin receptor-associated protein. J Biol Chem 1991, 266:13364–13369
24. Orlando RA, Kerjaschki D, Kurihara H, Biemesderfer D, Farquhar MG: gp330 associates with a 44-kDa protein in the rat kidney to form the Heymann nephritis antigenic complex. Proc Natl Acad Sci 1992, 89: 6698–6702
25. Kounnas MZ, Argraves WS, Strickland DK: The 39-kDa receptor-associated protein interacts with two members of the low density lipoprotein receptor family, α2-macroglobulin receptor and glycoprotein 330. J Biol Chem 1992, 267:21162–21166
26. Herz J, Goldstein JL, Strickland DK, Ho YK, Brown MS: 39-kDa protein modulates binding of ligands to low density lipoprotein receptor-related protein alpha 2-macroglobulin receptor. J Biol Chem 1991, 266: 21232–21238
27. Gutmann E, Niles J, McCluskey RT, Brown D: Colchicine-induced redistribution of an endogeneous apical membrane glycoprotein (gp330) in kidney proximal tubule epithelium. Am J Physiol 1989, 257: c397–c407
28. Collawn J, Paterson Y: Production of antipeptide antibodies. Current Protocols in Molecular Biology, 19th supplement. Edited by Ausubel F, Brent R, Kingston R, Moore D, Seidman J, Smith JA, Struhl K. New York, John Wiley and Sons, 1992, pp 11.15.1–11.15.4
29. Klassen J, Sugisaki T, Milgrom F, McCluskey RT: Studies on multiple renal lesions in Heymann nephritis. Lab Invest 1971, 25:577–585
30. McLean I, Nakane P: Periodate-lysine paraformaldehyde fixative: a new fixative for immunoelectron microscopy. J Histochem Cytochem 1974, 22:1077–1083
31. Altman L, Schneider B, Papermaster D: Rapid embedding of tissues in lowicryl K4M for immunoelectron microscopy. J Histochem Cytochem 1984, 32:1217–1223
32. Collins AB: Immunofluorescence. Diagnostic Immunopathology. Edited by Colvin RB, Bhan AK, McCluskey RT. New York, Raven Press, 1988, pp 421–435
33. Tokuyasu K: Immunocytochemistry on ultrathin frozen sections. Histochem J 1980, 12:381–403
34. Slot J, Geuze H: A new method for preparing gold probes for multiple labeling cytochemistry. Eur J Cell Biol 1985, 38:87–93
35. Kamata K, Baird L, Erikson M, Collins M, McCluskey RT: Characterization of antigens and antibody specificities involved in Heymann nephritis. J Immunol 1985, 135:2400–2408
36. Niles J, McCluskey RT, Ahmad MF, Arnaout MA: Wegener's granulomatosis autoantigen is a novel serine pioteinase. Blood 1989, 1888–1893
37. Selden R: Transfection using DEAE-Dextran. Current Protocols in Molecular Biology, 19th supplement. Edited by Ausubel F, Brent R, Kingston R, Seidman J, Smith JA, Struhl K. New York, John Wiley and Sons, 1992, pp 9.2.1–9.2.6
38. Moestrup S, Gliemann J: Purification of the rat hepatic alpha 2-macroglobulin receptor as an approximately 440-kDa single chain protein. J Biol Chem 1989, 264: 15574–15577
39. Goldstein JL, Brown M: Familial hypercholersterolemia. Metabolic basis of inherited disease, 5th ed. Edited by Stanbury J, Wyngaarden J, Fredrickson D, Goldstein J, Brown M. New York, McGraw-Hill Book Co., 1983, pp 672–712
40. Sahali D, Mulliez N, Chatelet F, Laurent-Winter C, Citadelle D, Roux C, Ronco P, Verroust P: Coexpression in humans by kidney and fetal envelopes of a 280 kDa-coated pit-restricted protein. Am J Pathol 1992, 140: 33–44
41. Herz J, Kowal RC, Goldstein JL, Brown MS: Proteolytic processing of the 600 kD low density lipoprotein receptor-related protein (LRP) occurs in a trans-Golgi compartment. EMBO J 1990, 9:1769–1776