IMMUNOCYTOCHEMICAL LOCALIZATION OF THE HEYMANN NEPHRITIS ANTIGEN (GP330) IN GLOMERULAR EPITHELIAL CELLS OF NORMAL LEWIS RATS*

By DONTSCHO KERJASCHKI‡ and MARILYN GIST FARQUHAR

From the Section of Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06510

Heymann's nephritis (HN)[1] is an experimental glomerulonephritis in rats which closely resembles membranous glomerulonephritis in humans. HN was originally induced by immunization of rats against autologous kidney cortex (1), and subsequently by cortical extracts, known as Fx1A, enriched in the nephritogenic antigen (2, 3), isolated microvilli of renal proximal tubules (4), and glycoproteins released therefrom by detergent or tryptic digestion (4). The hallmark of the disease is that anti-brush-border antibodies, either induced by active immunization of rats (active HN) or transferred to normal rats by intravenous injection (passive HN), are deposited in the lamina rara externa of the glomerular basement membrane (GBM) in a granular pattern (5–9) identical to that of the immune deposits in human membranous glomerulonephritis.

The deposition of antibodies directed against a tubular antigen in the glomerulus has been explained in several ways. For some years it was widely believed that it occurs as a result of the trapping of circulating antigen-antibody complexes in the GBM (10–12). However, recent results obtained by perfusion of isolated kidneys (8, 9, 13–15) have clearly indicated that there is direct binding of circulating antibodies to a fixed glomerular antigen. Results of immunocytochemical studies have corroborated the glomerular location of the antigen, but have provided conflicting data regarding where in the glomerulus (GBM, epithelial, or endothelial cell surfaces) the antigen is located (7, 9, 13, 15).

We have recently isolated and purified the nephritogenic antigen of HN from isolated kidney tubular microvillus membranes (16), and have identified it as a glycoprotein, M_r 330,000 (gp330). The availability of the purified antigen made it possible to make affinity-purified rabbit polyclonal and monoclonal anti-gp330 antibodies and to use these probes to localize the HN antigen in glomeruli of normal Lewis rats (a rat strain known to be very susceptible to the development of active HN) by immunocytochemistry. We here report our findings obtained by indirect immunoperoxidase procedures that demonstrate the exclusive localization of gp330

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[1] *Abbreviations used in this paper*: BSA, bovine serum albumin; DAB, diaminobenzidine; DFP, diisopropylfluorophosphate; DOC, sodium deoxycholate; DME, Dulbecco's minimal essential medium; ER, endoplasmic reticulum; GBM, glomerular basement membrane; gp330, the nephritogenic antigen of HN (16); HN, Heymann's nephritis; MEM, minimum essential medium; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; RIPA buffer, 0.1% SDS, 1% DOC, 1% Triton X-100, 150 mM NaCl, 25 mM Tris HCl, pH 7.2, and 10 mM EDTA; SDS, sodium dodecyl sulfate.

to glomerular epithelial cells where it is associated with several cell structures—endoplasmic reticulum (ER) and Golgi components, multivesicular bodies, and coated pits at the cell surface. The glomerular localization of gp330 has also been confirmed by immunoprecipitation of authentic gp330 from detergent extracts of biosynthetically labeled, isolated glomeruli.

Materials and Methods

Materials. Complete and incomplete Freund's adjuvant were obtained from Difco Laboratories, Detroit, MI; Alu-Gel S was obtained from SERVA Feinbiochemica GmbH & Co., Heidelberg, Germany; and rabbit anti-mouse IgG, specific for mouse immunoglobulin (Ig)G_1, IgG_2, IgG_3, and IgM were obtained from Bionetics Laboratory Products, Litton Bionetics Inc., Kensington, MD. F(ab) fragments of goat anti-rabbit IgG conjugated to rhodamine, $F(ab')_2$ fragments of goat anti-mouse IgG conjugated to fluorescein, and rabbit anti-mouse IgG were purchased from N. L. Cappel Laboratories Inc., Cochranville, PA, and F(ab) fragments of sheep anti-rabbit IgG conjugated to horseradish peroxidase were obtained from the Institute Pasteur Productions, Marnes La Coquette, France. Sodium dodecyl sulfate (SDS), acrylamide, Bio-Gel A 5m (100–200 mesh), and DEAE-Bio-Gel A were obtained from Bio-Rad Laboratories, Richmond, CA; lentil-lectin Sepharose CL-4B, protein A-Sepharose CL-4B, and cyanogen bromide-activated Sepharose 4B, from Pharmacia Fine Chemicals, Piscataway, NJ; [^{35}S]methionine and carrier-free ^{125}I, from Amersham Corp. Arlington Heights, IL; Dulbecco's minimal essential medium (DME), methionine-free minimum essential medium (MEM) (prepared from a kit), and penicillin-streptomycin solution, from Grand Island Biological Co., Grand Island, NY; diisopropyl fluorophosphate (DFP) and sodium deoxycholate (DOC), from Calbiochem-Behring Corp., San Diego, CA. *Clostridium perfringens* neuraminidase (type X, chromatographically pure), diaminobenzidine hydrochloride (type II), and α-methyl mannoside were purchased from Sigma Chemical Co., St. Louis, MO; En^{3}Hance, from New England Nuclear, Boston, MA; and Teflon screens (70, 150, and 350 μm mesh), from Swiss Silk Bolting Cloth Mfg. Co., Thal, Switzerland.

Purification of gp330. Gp330 was purified as described previously (16). Briefly, proximal tubule microvilli were prepared from kidneys of Sprague-Dawley rats (Charles River Breeding Laboratories, Inc., Wilmington, MA) by the Ca^{++}-precipitation method of Malathi et al. (17), and the membrane proteins were extracted therefrom with DOC-Tris buffer (1% DOC in 50 mM Tris-HCl, pH 8.6) and fractionated on a Bio-Gel A 5m (100–200 mesh) column. The fractions close to the void were pooled and bound to a lentil-lectin Sepharose CL-4B column. Contaminants were eluted with 10 mM α-methyl mannoside, and gp330 was eluted with 0.5 M α-methyl mannoside in DOC-Tris buffer containing 6 M urea and 10 mM EDTA. The eluate consisted of a single band with a mobility of ~330 kD as determined by SDS-polyacrylamide gel electrophoresis (PAGE). When injected into rats, it induced active HN (16).

Preparation of Rabbit Antibodies to gp330. A rabbit was immunized against purified gp330 following the immunization schedule of Louvard et al. (18). Initial immunization was by injection of 33 μg gp330, emulsified in complete Freund's adjuvant, into the popliteal lymph nodes and intradermally into the back. 21 d after the first injection, the animal received another 33 μg of antigen in incomplete Freund's adjuvant by subscapular injection. 10 d later the animal was given an intramuscular injection of 30 μg gp330 in phosphate-buffered saline (PBS), followed by an intravenous injection of 40 μg of antigen on the next day. Blood was collected at weekly intervals beginning with the 10th d after the final boost.

Rabbit IgG was purified on protein A-Sepharose CL-4B. To prepare affinity-purified anti-gp330 IgG, 250 μl rabbit serum was circulated over a column containing 500 μg purified gp330 immobilized to Sepharose 4B by cyanogen bromide. IgG was eluted with 0.1 M acetic acid in 0.9% NaCl, neutralized immediately with 1 M phosphate buffer, pH 7.2, and concentrated and dialyzed by vacuum dialysis. The specificities of the IgG were tested by immunoprecipitation and immunofluorescence.

Preparation of Monoclonal Antibodies to gp330. A male BALB/c mouse was immunized intraperitoneally with 30 μg of purified gp330 suspended in 0.5 ml PBS and emulsified in 0.5 ml Alu-Gel S. After 7 wk, the mouse was boosted intravenously with 30 μg gp330 in 0.25 ml PBS.

3 d thereafter spleen cells from the immunized mouse were fused with P3/NS 1/1-Ag4-1 mouse myeloma cells (obtained from Dr. J. Lin, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY) by the procedure of Kennett et al. (19). After 6 d, the supernatants of the resulting hybridomas were screened by indirect immunofluorescence for the presence of IgG, which binds to the brush borders of renal proximal tubules. Hybridomas that showed brush-border staining were cloned twice in soft agar, and the supernatants were screened as above. 60 positive IgG-producing clones were obtained. The IgG subclass was determined by double immunodiffusion against mouse IgG, subclass-specific antisera. Culture media from 10 of the positive clones were mixed and used as a monoclonal cocktail. One clone, D_1-$55F_2$1 (which produced an IgG_2), was expanded in mouse ascites. IgG was purified by ammonium sulfate precipitation and ion-exchange chromatography on DEAE-Bio-Gel A (20).

Perfusion of Kidneys for Biosynthetic Labeling with [^{35}S]Methionine. Rat kidneys were perfused as follows: The aorta distal to the left renal artery was exposed and cannulated, and the aorta was ligated between the two kidney arteries, after which the renal vein was opened (21). Perfusion pressure was kept at 120 mm Hg (flow rate ~10 ml/min). The cannula was placed in the aorta close to the opening of the left renal artery, and all small arterial vessels were carefully ligated. This procedure usually took 3–5 min, during which the kidney was continuously perfused with DME. The kidney was then removed and connected to a small recirculating perfusion apparatus (22), 50-ml capacity, equipped with a mechanical pump delivering 15 ml of oxygenated perfusate/min. A 0.22-μm Millipore filter (Millipore Corp., Bedford, MA) was placed immediately before the kidney to remove any circulating debris.

Kidneys were perfused for 20 min with oxygenated DME without methionine, which contained 100 mg/ml streptomycin and 100 U/ml penicillin. Two mCi of [^{35}S]methionine were then added, and perfusion was continued for 4 h. The labeling was ended by changing the perfusate to 50 ml of MEM, which contained a 10-fold excess of cold methionine and 2 mM DFP (to inhibit proteases).

Preparation of Glomerular and Brush-Border Subfractions. Glomeruli were separated from tubules by pressing minced kidney cortex successively through Teflon screens (350- and 150-μm pore width). They were subsequently collected on a smaller mesh screen (70 μm) and resuspended in DME containing 0.1% bovine serum albumin (BSA). Because the glomerular fractions always contained some (5–10%) tubular fragments, glomeruli were individually collected with glass pipettes under a dissecting microscope to avoid tubular contaminants. The glomeruli recovered from one kidney were pooled, pelleted in a Beckman microfuge (Beckman Instruments, Inc., Fullerton, CA), and extracted with 0.25% Triton X-100 in 25 mM Tris-HCl buffer, pH 7.2, for 20 min at 20°C with shaking. The extract was separated from the residue by sedimentation (10 min at 8,000 *g*) in the microfuge. The detergent concentrations of the supernatant were adjusted to that of the RIPA buffer used for immunoprecipitation (see below). The pellets were fixed for electron microscopy.

Purified brush-border microvilli were prepared (17) from the residual renal cortex remaining after the isolation of the glomeruli. The microvillus membranes were solubilized in a modified RIPA buffer (0.1% SDS, 1% DOC, 1% Triton X-100, 150 mM NaCl, 25 mM Tris HCl, pH 7.2, and 10 mM EDTA [23]) for 20 min at 20°C, and then centrifuged at 36,000 *g* for 20 min, as described previously (16).

Immunoprecipitation. Immunoprecipitations were carried out on (*a*) solubilized (in RIPA buffer) brush-border fractions labeled biosynthetically with [^{35}S]methionine or radioiodinated by the lactoperoxidase-glucose oxidase method (24) as previously described (16); and (*b*) Triton X-100 extracts of [^{35}S]methionine-labeled, isolated glomeruli. When polyclonal antibodies were used for immunoprecipitation, 50 μg of rabbit anti-gp330 IgG purified on protein A-Sepharose was incubated with 100,000 cpm of ^{125}I-labeled, or 60,000 cpm of [^{35}S]methionine-labeled, glomerular, or microvillar extracts for 1 h at 20°C, followed by a 1-h incubation with 100 μl protein A-Sepharose 4B (preswollen in RIPA buffer).

When monoclonal antibodies were used, extracts were similarly incubated with 25 μg monoclonal IgG purified from the ascites of clone, $D_1$55$F_2$1, or a mixture of the culture media of 10 different monoclonal strains, which contained 25 μg IgG. Rabbit anti-mouse IgG (75 μg) was used as a second antibody, and the resulting immune complexes were bound to protein A-Sepharose 4B, as was done for precipitations with polyclonal IgG.

SDS-PAGE. The immune complexes were released from the protein A-Sepharose by boiling them in 60 μl, 2 × concentrated SDS-PAGE sample buffer (final concentration: 3.65% SDS, 18 mM dithiothreitol, 4.5 mM EDTA, 6 M urea, and 10% glycerol) for 5 min. They were then subjected to electrophoresis for 6 h at 15 mA on gradient (3.6–8%) SDS-polyacrylamide gels with 6 M urea in a Maizel buffer system (25). The gels were fixed and stained with 0.2% Coomassie Blue R in 50% methanol/7% acetic acid. Gels containing ^{125}I-labeled material were dried and then autoradiographed on Kodak XO-mat RP-5 x-ray film (Eastman Kodak Co., Rochester, NY) by using a Dupont Cronex intensifying screen (DuPont Instruments, Wilmington, DE) at −70°C. [^{35}S]methionine-labeled material was visualized by fluorography, soaking the gels in En^{3}Hance before exposing the x-ray film at −70°C.

Perfusion and Fixation of Kidneys for Immunocytochemistry. The left kidneys of Lewis rats were perfused for 3 min with 25 ml MEM at 20°C via the aorta, as described above. After flushing, small pieces of the unfixed, perfused kidney cortex were prepared and snap frozen in isopentane (−160°C), cooled, and stored in liquid nitrogen (26) until used for immunofluorescence. Other kidneys were flushed with DME and perfusion fixed with 50 ml of aldehyde fixative (4% freshly prepared formaldehyde and 0.1 or 0.5% glutaraldehyde in 100 mM cacodylate buffer, pH 7.2) for 5 min at 20°C, followed by perfusion with PBS containing 50 mM glycine for 10 min (to end fixation and to quench residual aldehyde groups). Tissue slices were transferred to PBS containing 10% dimethyl sulfoxide frozen in isopentane as was done for unfixed tissue, and stored in liquid nitrogen until used for immunofluorescence or immunoperoxidase.

Localization of gp330 in Normal Rats by Indirect Immunofluorescence. Sections (2–4 μm) of unfixed kidney prepared on a cryostat and thin (0.5–1 μm) frozen sections of aldehyde-fixed kidney tissue prepared (27) on a Sorvall MT-2B ultramicrotome equipped with an LTC.2 adapter for ultracryomicrotomy (DuPont Instruments-Sorvall, DuPont Co., Newtown, CT) were processed for indirect immunofluorescence. The sections were first incubated for 30 min at 20°C with (*a*) rabbit anti-gp330 serum; (*b*) IgG purified on protein A-Sepharose (25 μg/ml); (*c*) rabbit affinity-purified anti-gp330 IgG (5 μg/ml); (*d*) anti-gp330 IgG purified from the ascites of mice inoculated with $D_1 55F_2 1$ hybridoma cells (10 μg IgG/ml); or (*e*) a mixture of the culture media of 10 different anti-gp330-secreting hybridoma clones (20 μg/ml). Sections that had been incubated with rabbit antibodies (*a–c*) were then exposed for 30 min at 20°C to rhodamine-conjugated goat anti-rabbit F(ab), whereas sections that had been incubated with mouse monoclonal antibodies (*d* and *e*) were exposed to fluorescein-conjugated goat anti-mouse F(ab')$_2$ as the second reagent. The latter conjugate was passed over a column with 25 mg purified rat IgG immobilized to cyanogen bromide-activated Sepharose 4B before use to eliminate cross-reactivity with rat IgG. In some experiments cryostat sections were digested with neuraminidase (15 U/ml in 25 mM acetate buffer, pH 5, for 30 min at 37°C) before antibody incubation. All sections were examined and photographed with a Zeiss photomicroscope II (Carl Zeiss, Inc., NY) equipped with epifluorescence optics and appropriate filters for rhodamine and fluorescein.

Localization of gp330 by Indirect Immunoperoxidase (Electron Microscopy). Cryostat sections (10–15 μm) were prepared from aldehyde-perfused kidneys and incubated in suspension (26, 28, 29) with affinity-purified, rabbit anti-gp330 IgG in PBS (5 μg/ml) that contained 0.1% BSA for 2 h at 20°C for 12 h at 4°C. After being washed in PBS, the sections were further incubated for 2 h at 20°C in peroxidase-conjugated sheep anti-rabbit F(ab) (diluted 1:20 in PBS) that contained 0.1% BSA. Sections were then washed and the peroxidase activity was visualized by incubation in 10 ml of 0.1% diaminobenzidine (DAB) in 50 mM Tris-HCl buffer, pH 7.2, for 2–10 min at 20°C. The DAB reaction was started by adding 0.1 ml of 1% H_2O_2 and was allowed to proceed for 1–15 min (29).

Sections were also incubated in the monoclonal antibody mixture and processed similarly, except that an additional step was necessary: After incubation with the first antibody, sections were exposed to rabbit anti-mouse IgG (50 μg/ml) for 2 h at 20°C before incubation in the peroxidase conjugate. In addition, some sections were treated with neuraminidase (as described for immunofluorescence) before incubation with either rabbit or monoclonal anti-gp330 IgG.

After the DAB reaction, sections were postfixed in OsO_4 reduced with potassium ferrocyanide (30), and embedded in Epon 812 as detailed previously (26, 28). Thick sections (0.5 μm) were prepared from Epon-embedded blocks on a Reichert ultracut ultramicrotome and examined

by light microscopy. Silver sections similarly prepared and examined in a Philips 301 electron microscope (Philips Electronic Instruments, Inc., Mahwah, NJ).

Induction of Passive HN in Rats by Injection of Rabbit Anti-gp330 IgG. Two Lewis rats were injected intravenously via the femoral vein with protein A-purified, anti-gp330 IgG (1 mg in 0.5 ml PBS) obtained from the rabbit immunized against gp330. After 3 d, the kidneys of the injected rats were either flushed with DME or aldehyde fixed by perfusion and frozen as described above. Cryostat sections (2–4 μm) prepared from unfixed tissue were incubated (30 min at 20°C) with rhodamine-conjugated goat anti-rabbit $F(ab')_2$ for direct immunofluorescence; cryostat sections (10–14 μm) prepared from aldehyde-fixed tissue were exposed to peroxidase-conjugated sheep anti-rabbit F(ab) (4 h at 20°C), incubated with DAB for direct immunoperoxidase, and embedded in Epon.

Controls. Controls for immunofluorescence and immunoperoxidase consisted of the omission of the first antibody, incubation with immune serum or IgG adsorbed against purified gp330, and incubation in DAB medium alone.

Results

Characterization of Anti-gp330 Antibodies

The specificity of the polyclonal and monoclonal antibodies was assessed by immunoprecipitation and immunofluorescence:

IMMUNOPRECIPITATION. Serum obtained from the rabbit immunized with gp330 specifically immunoprecipitated gp330 from ^{125}I-labeled or [^{35}S]methionine-labeled (Fig. 1, lane g) solubilized renal cortical microvilli.

Similarly, the serum of the mouse whose spleen was used for fusion (Fig. 1, lane c),

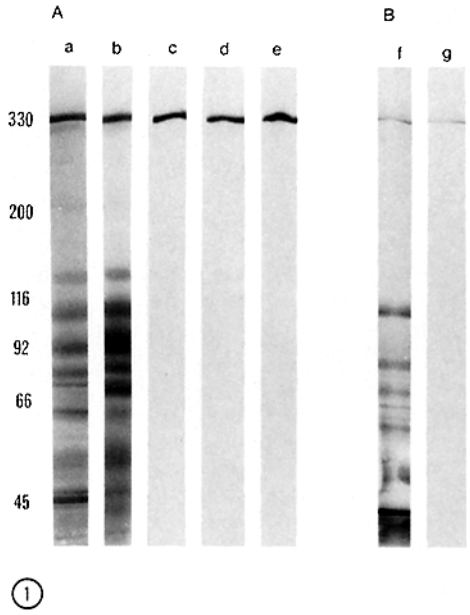
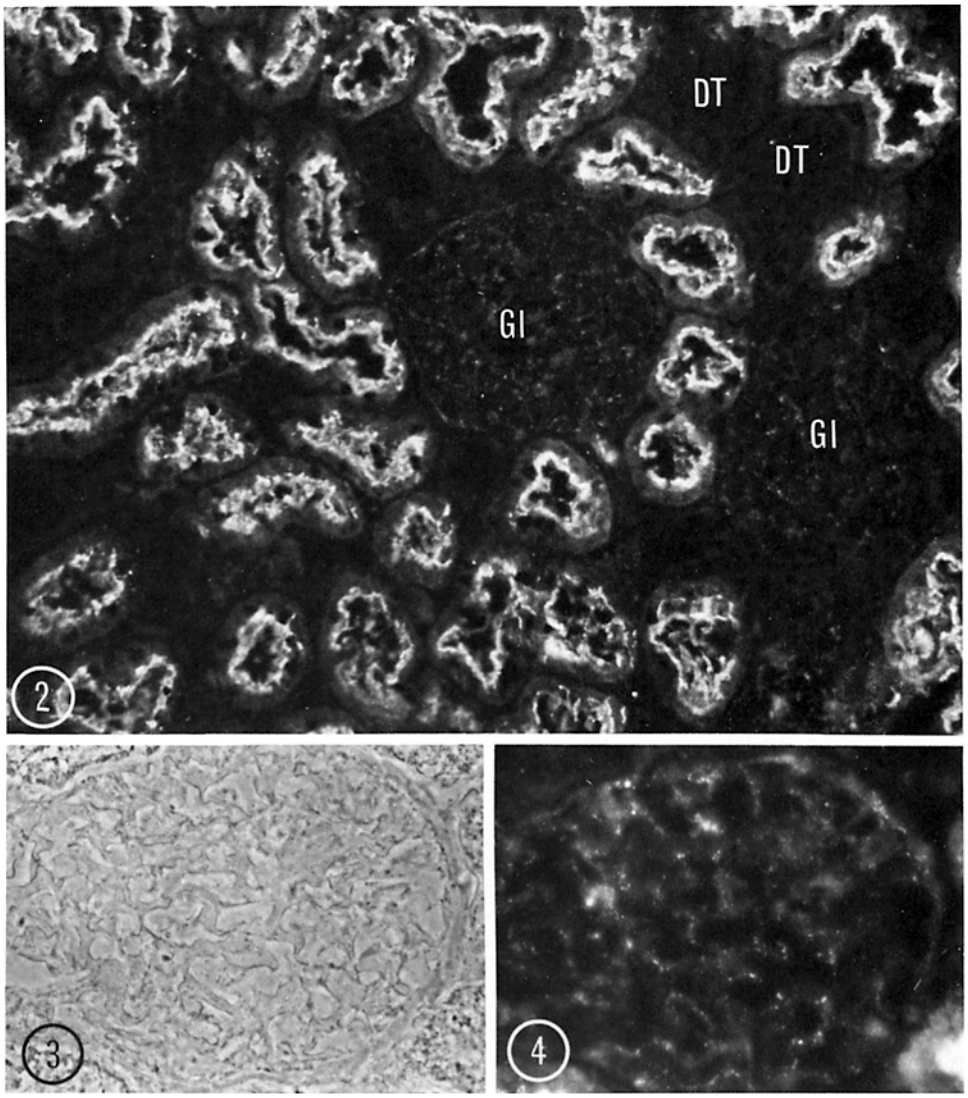


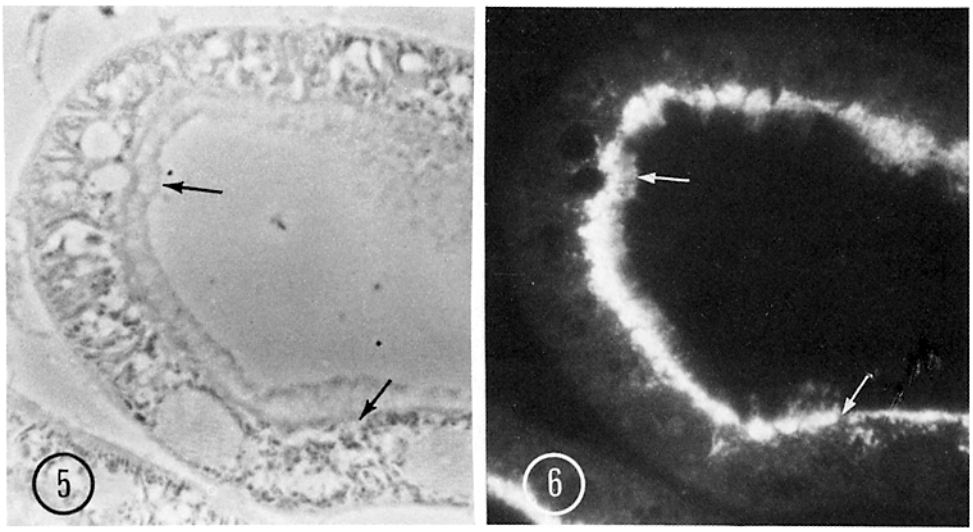
FIG. 1. SDS-PAGE analysis of immunoprecipitates obtained with ^{125}I-labeled (A) or [^{35}S]methionine-labeled (B) microvillus membranes and anti-gp330 antibodies. *Lane a*: Coomassie Blue-stained preparation of solubilized microvillus membranes. *Lane b*: Autoradiogram of ^{125}I-labeled solubilized microvillus membranes, used as the starting material for immunoprecipitation. *Lanes c–e*: Immunoprecipitates obtained from *b* with, respectively, serum of the mouse used for fusion with spleen cells, IgG produced by clone $D_1 55F_2 1$, and a mixture of the culture supernatants of 10 different clones. *Lane f*: Fluorogram of [^{35}S]methionine-labeled microvillus membranes. *Lane g*: Immunoprecipitate obtained from *f* with purified (on protein A-Sepharose) rabbit anti-gp330 IgG. In all cases only a band corresponding to gp330 is precipitated.



FIGS. 2–4. Indirect immunofluorescence preparation of unfixed cryostat sections from a normal Lewis rat kidney incubated with affinity-purified polyclonal anti-gp330 IgG. Fig. 2 shows that the brush borders of proximal tubules are heavily stained, whereas distal tubules (DT) are unstained. Two glomeruli (Gl) show a faint granular staining. Figs. 3 and 4 are phase-contrast and immunofluorescent micrographs, respectively, of a glomerulus. By comparing the two images it can be deduced that the diffuse fluorescent staining is localized outside the capillaries. Fig. 2, × 380; Figs. 3 and 4, × 700.

the culture fluid mixture obtained from different hybridoma clones (Fig. 1, lane e), and the purified monoclonal IgG prepared from the ascites fluid of clone $D_1 55 F_2 1$ (Fig. 1, lane d), precipitated only gp330 from solubilized, radioiodinated microvillus membranes.

BRUSH-BORDER STAINING. Both the polyclonal and monoclonal antibodies specifically stained brush borders of the proximal tubules as determined by indirect



FIGS. 5 and 6. Phase-contrast and immunofluorescence micrographs of a portion of a proximal tubule from a 0.5-μm frozen section incubated with monoclonal anti-gp330 IgG. Upon comparing the two images it is evident that the microvilli of the tubular brush border (upper arrow) are intensely stained, and that an even more intense granular staining is seen at the base of the microvilli (lower arrow), in a location corresponding to that of coated pits or coated invaginations of the plasmalemma (see Fig. 7). × 800.

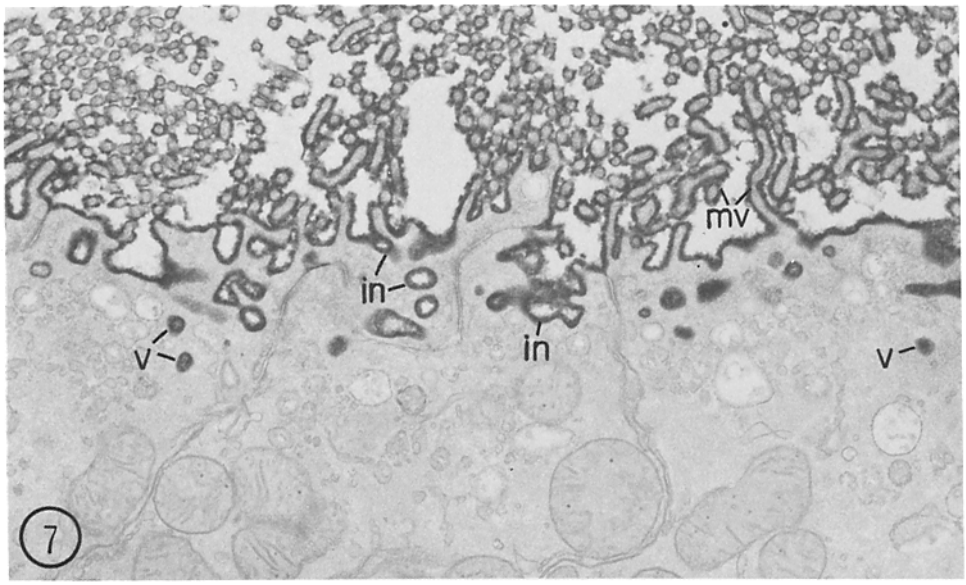


FIG. 7. Indirect immunoperoxidase staining of a proximal tubule cell with monoclonal anti-gp330 IgG (neuraminidase-pretreated section). DAB-reaction product is seen on the microvilli (mv), in coated invaginations at the base of the microvilli (in), and in coated vesicles (v) located in the apical region of the cell. × 26,000.

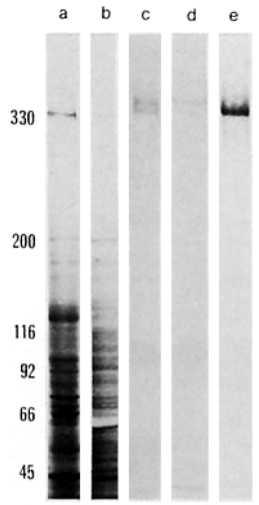


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FIG. 8. SDS-PAGE fluorographic analysis of immunoprecipitates prepared from [^{35}S]methionine-labeled microvillus fractions (lanes a and e) and glomerular fractions (lanes b–d). *Lane a*: Solubilized microvillus membrane fraction. *Lane b*: Triton X-100 extract of isolated glomeruli. *Lanes c and d*: Immunoprecipitates obtained from *b* with a monoclonal IgG mixture and rabbit anti-gp330 IgG, respectively. *Lane e*: Immunoprecipitate of *a* obtained with the mixed monoclonal anti-gp330 IgG. Note that only one band is precipitated from the microvillar fraction *e*, whereas two bands, one of which is believed to represent a precursor of gp330, are precipitated from glomerular fractions *c* and *d*.

immunofluorescence of unfixed (Fig. 2) or fixed (Fig. 6) cryostat sections of normal Lewis rat kidneys. Identical results were obtained with whole serum, IgG purified on protein A-Sepharose 4B, affinity-purified IgG from the rabbit immunized with anti-gp330, monoclonal antibodies obtained from the ascites fluid of clone $D_1 55 F_2 1$, or the culture fluid mixture obtained from 10 different hybridoma clones. In keeping with a recent report by Ehrlich et al. (31), the fluorescent signal obtained with the mixture of 10 monoclonal antibodies was consistently higher than that obtained with a single monoclonal IgG, and therefore the mixture was used for subsequent immunocytochemistry.

With the increased resolution achievable in the 0.5-μm ultracryomicrotomy sections (Figs. 5 and 6), it could be seen that the staining is especially concentrated at the base of the microvilli in the location corresponding to the coated pits. In sections incubated for immunoperoxidase and examined by electron microscopy, it is clear that the DAB reaction is found on the outside of the microvillar membranes and is especially concentrated in the coated invaginations located at the base of the microvilli (Fig. 7).

Immunoprecipitation of gp330 from Isolated Glomeruli. Glomeruli were individually selected by hand in order to avoid tubular contamination. When such handpicked glomeruli from kidneys biosynthetically labeled with [^{35}S]methionine were solubilized and used for immunoprecipitation, both rabbit anti-gp330 and the monoclonal anti-gp330 IgG mixture specifically precipitated gp330 (Fig. 8, lanes c and d). The extraction procedure solubilized most membrane material, and left behind the GBM,

cytoskeletal elements, heterochromatin, and the slit diaphragms, as seen in the residual pellet by electron microscopy (not shown).

Localization of gp330 in the Glomerulus by Light Microscopy. By indirect immunofluorescence, both the polyclonal rabbit anti-gp330 IgG and the monoclonal anti-gp330 IgG stained the glomerulus in a faint but distinct granular pattern (Figs. 2 and 4). The stained dots, which were usually so small that they were at the limit of the optical resolution, varied in their fluorescence intensity, but their staining was much less intense than that of the microvilli of adjacent proximal tubules (Fig. 2). The distribution of the staining in glomeruli appeared to correspond to that of the epithelium (Fig. 4). When sections were digested with neuraminidase before exposure to the antibodies, the fluorescent pattern was identical to that seen in unpredigested material.

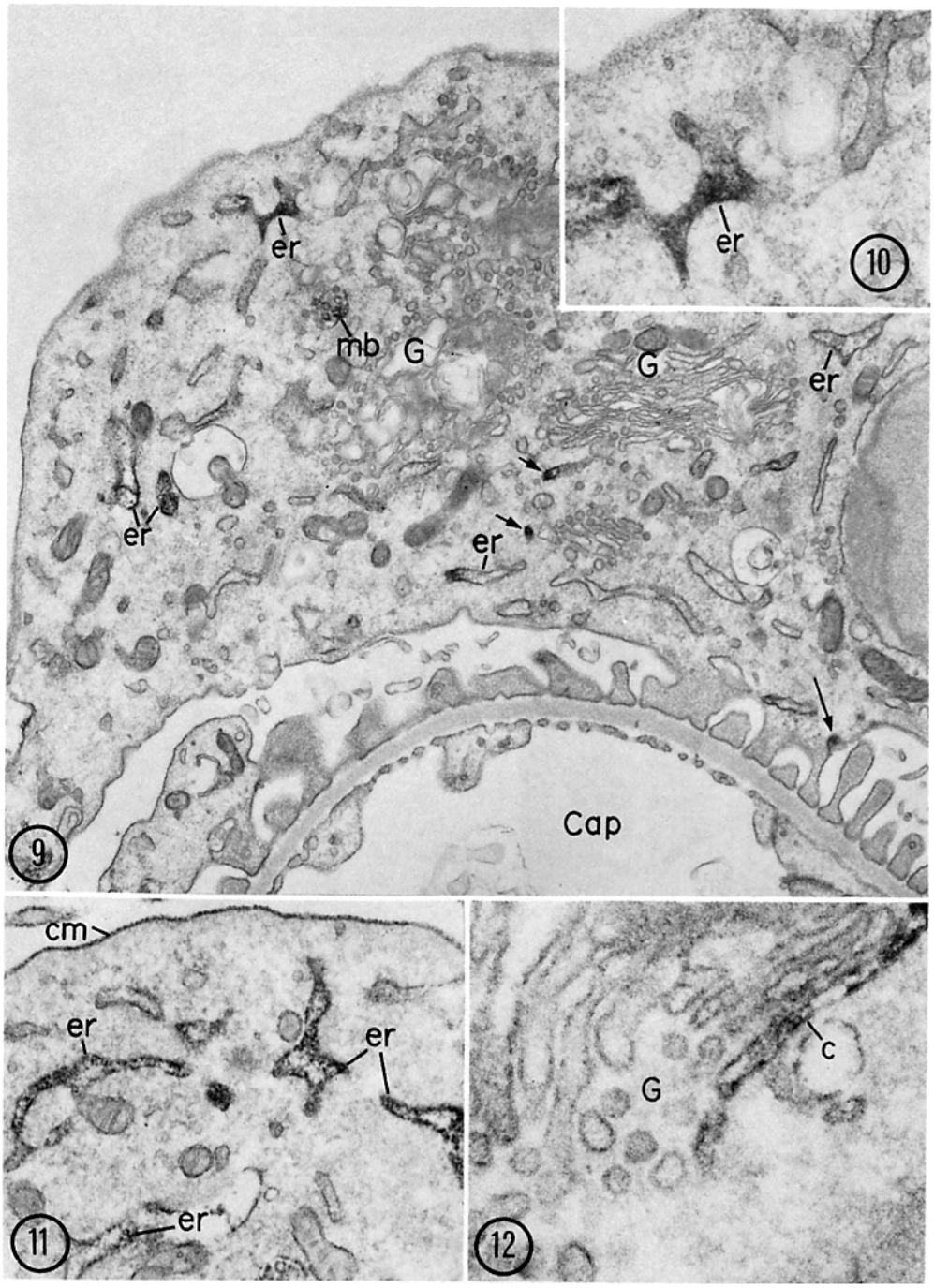
When 1-μm plastic sections cut from Epon-embedded blocks of kidneys processed for immunoperoxidase were examined, it was evident that the reaction product was associated exclusively with epithelial cells. No staining of mesangial or endothelial cells was seen (see Fig. 16). There was no staining of glomeruli or the tubules in any of the controls.

Ultrastructural Localization of gp330 in Glomeruli by Immunoperoxidase

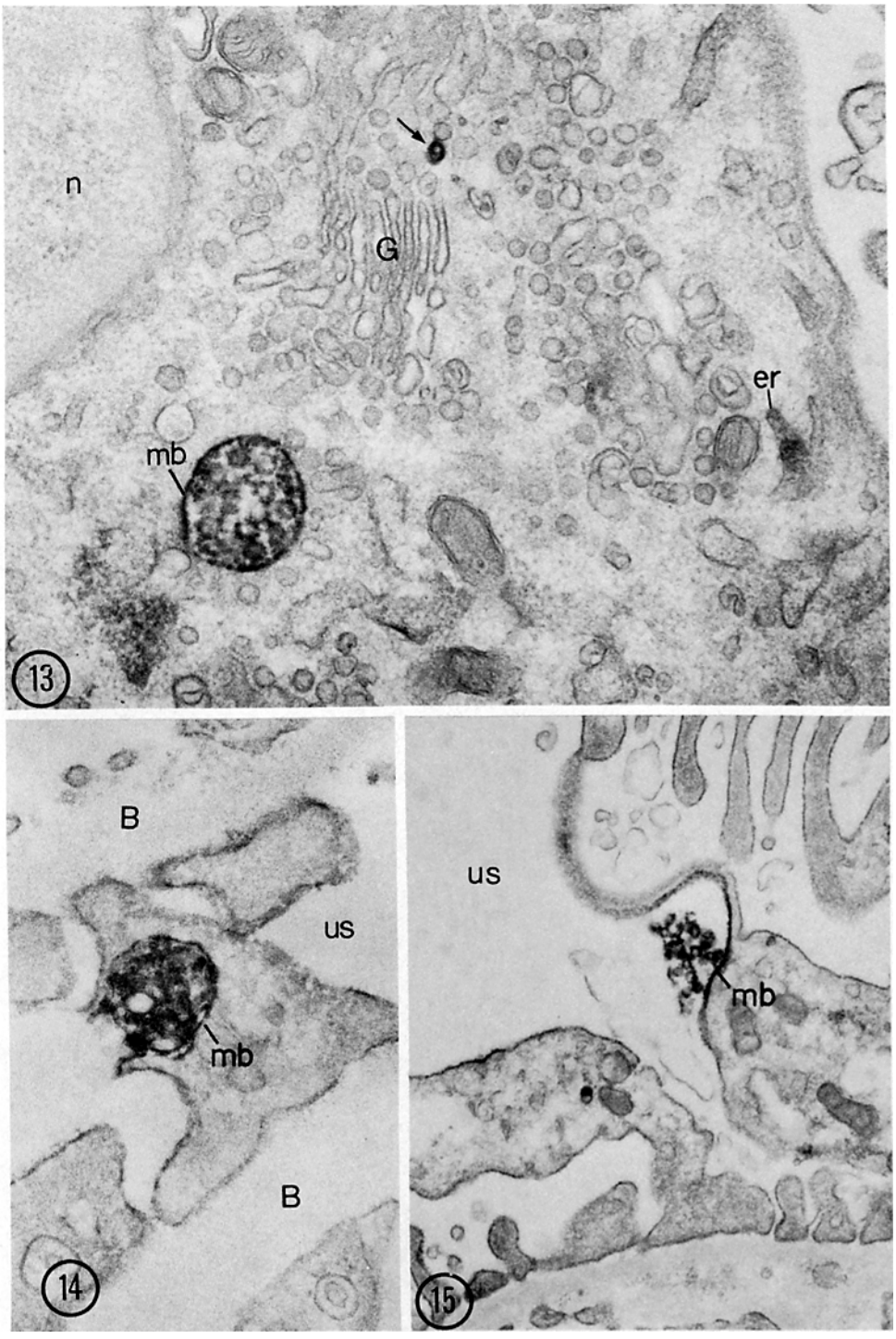
Electron microscopy confirmed that when either affinity-purified rabbit anti-gp330 antibody, or a mixture of monoclonal anti-gp330 IgG were used for the localization, reaction product was restricted to glomerular epithelial cells and was not found in or on endothelial cells, mesangial cells, or the GBM. The data obtained with both types of antibodies were identical.

LOCALIZATION OF GP330 WITHIN ORGANELLES OF THE GLOMERULAR EPITHELIAL CELLS. Reaction product was detected in several intracellular locations: in some but not all cisternae of the ER, in some Golgi elements, and in multivesicular bodies (Figs. 9–15). Reactive ER cisternae could be identified on the basis of their characteristic location and morphology (Figs. 9–11). Ribosomes were not clearly demonstrable on the ER membranes because of the use of reduced OsO_4, a procedure which produces good membrane staining but low contrast of ribosomes. The Golgi-associated reaction product was localized mainly within vesicles on the *trans* side of this organelle (Fig. 9). Staining of one or two of the stacked cisternae was also observed occasionally (Fig. 12). Reaction product was found in most but not all multivesicular bodies where it filled the matrix and spared the vesicles (Fig. 13). These bodies were located in both the epithelial perikarya and foot processes. Occasionally, multivesicular bodies were encountered that were open at the cell surface, apparently captured in the process of discharging, by exocytosis, their positively stained contents into the urinary spaces (Figs. 14 and 15). Exocytosis of lysosomal or prelysosomal structures (dense bodies and multivesicular bodies) is a frequent finding in the glomerular epithelium, especially in aminonucleoside-nephrotic animals where there is overloading of the lysosomal system due to amplified protein absorption (32).

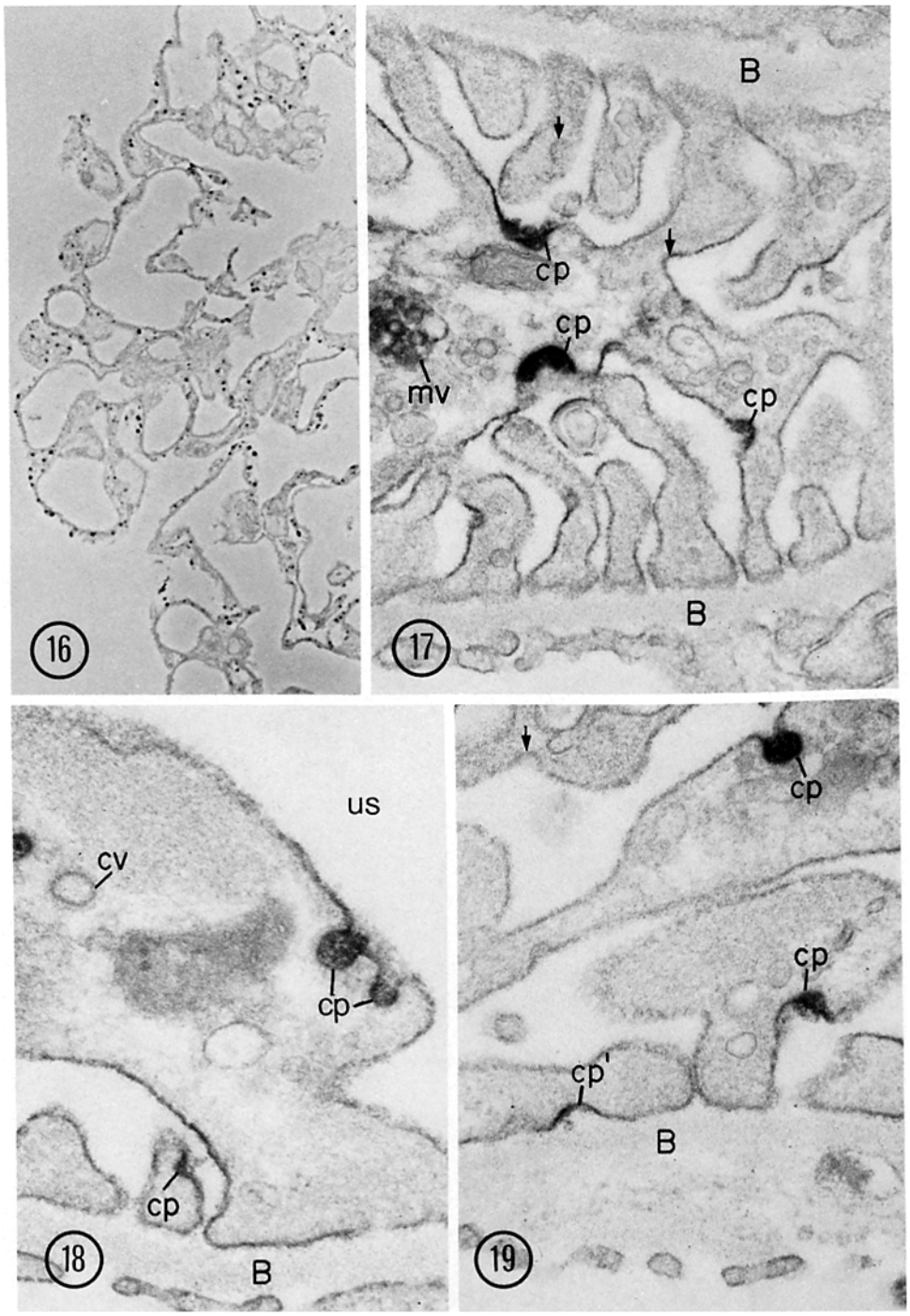
LOCALIZATION OF GP330 IN COATED PITS AT THE EPITHELIAL CELL SURFACE. Reaction product was concentrated in numerous invaginations of the epithelial cell membrane facing the urinary spaces. Typically these positively stained components possessed a bristle coat on their cytoplasmic aspect (Figs. 17 and 18), identical to that of clathrin-coated vesicles and pits in other locations (33, 34). In sections not pretreated with neuraminidase, such stained coated pits were seen all along the epithelial cell surface,



FIGS. 9–12. Detection of gp330 by indirect immunoperoxidase in glomerular epithelial cells (normal Lewis rat kidney). Fig. 9 is a low-power view showing peroxidase reaction product in several cisternae of the endoplasmic reticulum (er), in a multivesicular body (mb) located in the Golgi (G) region, in elements (short arrows) of the Golgi complex (G), and in an invagination of the plasmalemmma (long arrow, lower right corner). Figs. 10 and 11 are enlargements showing peroxidase-reaction product in several ER cisternae (er). A thin layer of reaction product covers the cell membrane (cm) of the epithelial cell in Fig. 11. Fig. 12 shows reaction product in one of the cisternae (c) of a Golgi stack (G). For Figs. 9, 10, and 12, affinity-purified rabbit anti-gp330 was used as first antibody. For Fig. 11, a monoclonal anti-gp330 IgG mixture was used. Fig. 9, × 9,200; Fig. 10, × 42,000; Fig. 11, × 36,000; Fig. 12, × 88,000.



FIGS. 13–15. Indirect immunoperoxidase staining with affinity-purified rabbit anti-gp330 IgG. Fig. 13 shows the Golgi region (G) of a glomerular epithelial cell with a multivesicular body (mb) and a vesicle (arrow) containing reaction product. An ER cisterna (er) also contains traces of reaction product. Figs. 14 and 15 show multivesicular bodies located in a foot process and a larger epithelial cell process that were apparently fixed while in the process of discharging their reactive content into the urinary spaces (us) by exocytosis. B, basement membrane; n, nucleus. Fig. 13, × 47,500; Fig. 14, × 57,200; Fig. 15, × 28,500.



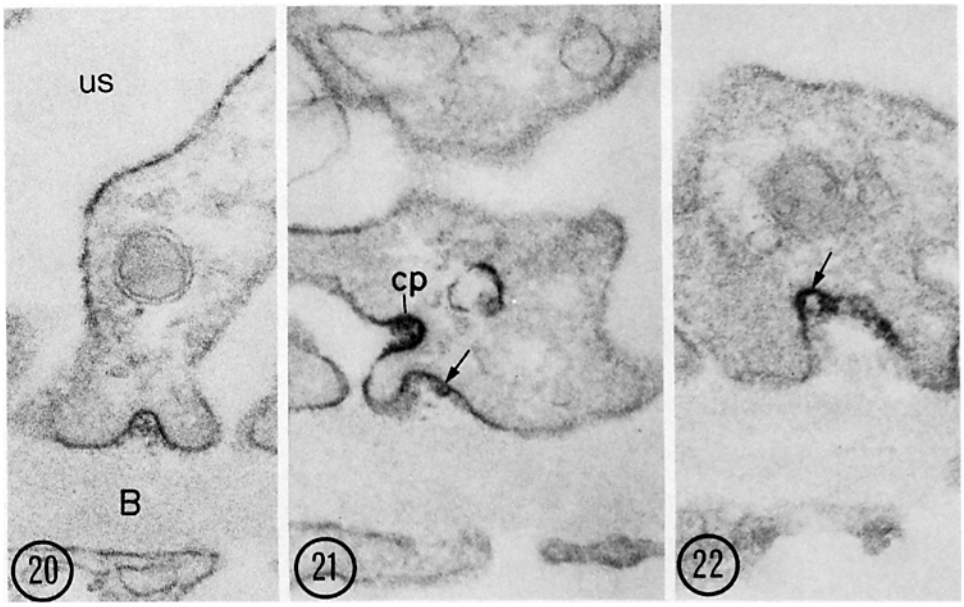
FIGS. 16–19. Indirect immunoperoxidase staining of normal Lewis rat kidney using affinity-purified rabbit anti-gp330 IgG and mouse monoclonal IgG (Fig. 17) as the first antibodies. Fig. 16 is a light micrograph taken from a 0.5-μm section prepared from an Epon-embedded block, demonstrating that DAB-reaction product is located outside of the capillary lumen and is restricted to glomerular epithelial cells. Figs. 17 and 18 show processes of epithelial cells with a number of pits (cp) which contain recognizable coats and are reactive for gp330. Other coated pits (arrows, Fig. 17) are not reactive. A faint staining is also seen on those portions of the cell membrane in close proximity to the reactive coated pits. Fig. 19 is from a sample that had been digested with neuraminidase before antibody incubation. Besides two reactive coated pits (cp) located on the epithelial cell processes, a membrane invagination that stains for gp330 can also be seen at the base of the foot processes (cp'). cv, coated vesicle. Fig. 16, × 700; Fig. 17, × 45,000; Fig. 18, × 57,800; Fig. 19, × 57,800.

including the sides of the foot processes, but not at their base. Not all of the pits present contained reaction product, and sometimes nonreactive and reactive coated pits were seen side by side (Fig. 17). Occasionally similar positively stained invaginations were seen on the membranes of the epithelium of Bowman's capsule.

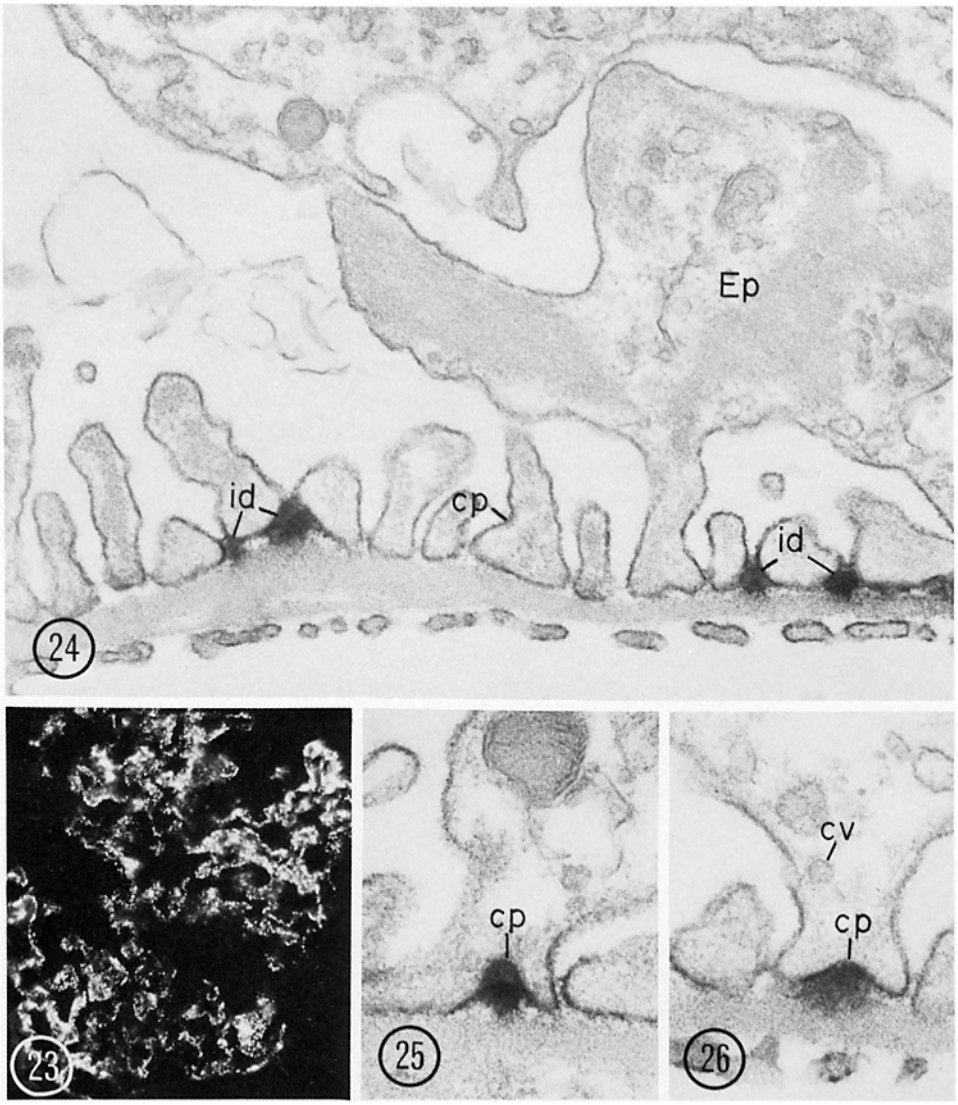
When sections were pretreated with neuraminidase before antibody incubation, reaction product was also seen in membrane invaginations at the base of the foot processes (Figs. 19–22). The reactive pits occurred all along that portion of the foot processes facing the GBM, i.e., near the slit diaphragm (Fig. 21) or away from it (Figs. 19 and 20). Coats could be identified on many of the pits. The ratio of nonreactive to reactive pits was higher and reaction product was generally less abundant for those located at the base of the foot processes than for those along the remaining cell surface.

MEMBRANE STAINING. In the neuraminidase-pretreated sections incubated with the monoclonal IgG mixture, the entire surface of the epithelial cell was covered by a thin layer of reaction product (Fig. 11). Cell membrane labeling was less pronounced and more patchy in neuraminidase-predigested sections incubated with rabbit anti-gp330. In the latter specimens membrane staining was often seen in the immediate vicinity of reactive pits (Figs. 17 and 18) and in the vicinity of discharging multivesicular bodies (Fig. 15). The latter localization could reflect the true distribution of the antigen, or could be due to local diffusion of DAB reaction product from sites of high reactivity (29).

CONTROLS. No staining was seen in any of the controls.



FIGS. 20–22. Indirect immunoperoxidase staining of sections digested with neuraminidase before incubation with affinity-purified rabbit anti-gp330 IgG. This gallery of figures shows localization of gp330 in invaginations at the base of the foot processes, facing the GBM (B). In Figs. 21 and 22 coats can be seen on the cytoplasmic aspect of the membrane indentations (arrows). In Fig. 21, a reactive coated pit (cp) is also present on the side of one of the foot processes. Fig. 20, × 78,000; Fig. 21, × 78,000; Fig. 22, × 72,000.



FIGS. 23–26. Localization of rabbit anti-gp330 IgG administered intravenously to a normal rat (passive HN). Cryostat sections of the kidney were stained by direct immunofluorescence (Fig. 23) or direct immunoperoxidase (Figs. 24–26) for the presence of rabbit IgG. Fig. 23 shows the fine granular distribution of rabbit IgG seen by immunofluorescence in a glomerulus. Fig. 24 shows the typical location of four immune deposits (id) containing rabbit anti-gp330 in the lamina rara externa under the filtration slits. Figs. 25 and 26 show small immune deposits, located in coated pits (cp) at the base of the foot processes and extending into the lamina rara externa of the GBM. cv, coated vesicle. Fig. 23, × 600; Fig. 24, × 31,000; Fig. 25, × 51,000; Fig. 26, × 51,000.

Ultrastructural Localization of Anti-gp330 IgG Injected into Normal Rats. When monospecific rabbit anti-gp330 IgG was injected intravenously into normal rats and their kidneys were examined 3 d later, fine granular deposits of IgG were seen in the glomeruli by direct immunofluorescence (Fig. 23). This pattern is considered the hallmark of passive HN and was identical to that induced previously by injection of rat anti-gp330 IgG (16).

By immunoperoxidase, small immune deposits that contained rabbit IgG were detected in the lamina rara externa of the GBM, located either immediately below the slit diaphragms (Fig. 24) or beneath the foot processes of the epithelial cells. A striking finding was that sometimes deposits of reaction product, which indicated the presence of anti-gp330 IgG, were located in invaginated pits at the base of the foot processes (Figs. 25 and 26). The latter were identical to the coated pits in which gp330 was localized (compare Figs. 20–22 with 25 and 26).

Discussion

In this investigation we have shown that gp330, previously demonstrated to be the brush-border component that is the pathogenic antigen of HN (16), is also a component of the glomerular epithelial cell of Lewis rat kidneys. By using immunoperoxidase immunocytochemistry and both polyclonal and monoclonal antibodies, gp330 was detected intracellularly in the rough ER, in Golgi elements, and in multivesicular bodies of this cell type. In addition, most conspicuously, it was detected in pits located all along the membrane of epithelial cells, including the domain facing the GBM. Many of these gp330-containing membrane indentations showed typical cage-like (clathrin) coats, and may thus be classified as coated pits (34). That treatment with neuraminidase was needed to expose anti-gp330 reactive sites located at the base of the foot processes suggests that the presence of sialic acid may normally render these sites inaccessible to the antibody molecules. The epithelial cell membrane is known to contain unusually high concentrations of sialic acid (35); the latter presumably represent the peripheral hexose moieties of intrinsic membrane glycoproteins or glycolipids.

The immunocytochemical data clearly demonstrate that antigenic sites that cross-react with gp330 are present in the glomerular epithelium. The possibility that the positive staining of the glomerular epithelium could be due to nonspecific binding of IgG, or to the presence of endogenous peroxidatic activity in these sites, has been ruled out by appropriate control experiments. The immunoprecipitation data show that a glycoprotein with a similar molecular weight (as determined by SDS-PAGE) to that of gp330 purified from brush-border membranes is present in glomeruli: Monospecific rabbit or monoclonal IgG precipitated gp330 from Triton-extracts of biosynthetically labeled glomeruli. The possibility that the gp330 precipitated from glomerular extracts was of tubular origin was effectively ruled out by using purified, hand-selected glomeruli free from tubular contamination as the star ing material for the immunoprecipitations.

The localization of gp330 in the rough ER of glomerular epithelial cells suggests that the HN antigen is produced by this cell type. The other sites of localization (Golgi elements, multivesicular bodies, and coated pits) could be explained by the following sequence of events: gp330, known to be a membrane-associated glycoprotein, is (*a*) synthesized by glomerular epithelial cells; (*b*) transported to the Golgi complex where terminal glycosylation is known to occur (36); (*c*) transported to the cell membrane in vesicles such as those seen near the stacked Golgi cisternae (Figs. 9 and 13); and (*d*) becomes concentrated in coated pits at the cell surface. This sequence is plausible because this is the biosynthetic route believed to be followed by membrane glycoproteins (36). Just how gp330 becomes associated with multivesicular bodies is not clear at present since these bodies, which are usually considered to be prelysosomal

structures, are known to be involved in the uptake of both intracellular (37) and circulating (38) proteins. It can be explained in several ways: (*a*) some of the newly synthesized gp330 is for unknown reasons segregated into multivesicular bodies rather than delivered to coated pits; (*b*) cell surface gp330 is internalized into multivesicular bodies; or (*c*) circulating gp330 produced elsewhere binds to coated pits, is taken up by endocytosis, and is subsequently delivered to the multivesicular bodies.

The reaction product seen along the epithelial cell membrane, after staining with monoclonal and polyclonal anti-gp330 IgG following neuraminidase treatment, could indicate the presence of gp330 on the plasmalemma in locations other than the coated pits, or it could result from diffusion of oxidized DAB from reactive sites and its readsorption onto the epithelial cell membrane. This type of DAB diffusion artifact has been demonstrated in immunoperoxidase preparations after prolonged incubation (29). Alternative immunocytochemical procedures are needed to resolve this issue.

Several previous attempts have been made to localize the HN antigen in glomeruli by immunocytochemistry. Results obtained by immunofluorescence, using either heterologous anti-Fx1A antibody (7–9) or IgG eluted from kidneys of rats with active HN (13–15), are in agreement that the antigen(s) recognized by these antibodies is present in the glomerulus, and that neuraminidase treatment of the sections before immunostaining enhances the glomerular signal (8, 13).

Results obtained at the electron microscope level with immunoperoxidase procedures are more conflicting. Van Damme et al. (8), using rabbit anti-Fx1A antibodies, found DAB reaction product on both glomerular epithelial and endothelial cell membranes, and throughout the GBM where it was distributed in a granular pattern. Neale and Wilson (13), using antibody eluted from the kidney cortex of animals with HN, localized peroxidase reaction product to the GBM where it was particularly prominent beneath the foot processes. Fleuren et al. (7), also using eluted antibody, found the main site of the reaction product to be the cell membrane at the base of the foot processes.

Our findings, using polyclonal and monoclonal IgG raised against the purified pathogenic protein, confirm the immunofluorescence findings obtained by others of a dispersed, fine, granular staining in glomeruli. Taking advantage of affinity-purified and monoclonal antibodies to gp330, we carried out immunoperoxidase localizations at the electron microscope level and obtained findings suggesting that gp330 is an indigenous glomerular antigen synthesized by the visceral epithelium. This conclusion is based on the localizaton of gp330 in intracellular compartments (ER and Golgi) involved in the synthesis of proteins for export. That the HN antigen is of epithelial origin was already suggested by the demonstration of Fx1A antigens in cultured glomerular epithelial cells (39).

Of particular interest in the present work is the novel finding that gp330 is concentrated in coated pits at the epithelial cell surface. The demonstration of the antigen in coated pits found along the entire glomerular epithelial cell surface as well as at the base of the proximal tubule microvilli raises the intriguing question of what it is doing in these structures, a topic about which we can only speculate at present. Since the main known function of coated plasmalemmal vesicles is in receptor-mediated endocytosis (34, 40, 41), its presence in these structures suggests its involvement, as either receptor or ligand, in receptor mediated transport processes.

The hallmark of HN, along with several human glomerular immune complex

diseases, is the formation of immune deposits in the lamina rara externa of the GBM. This peculiar pattern, originally believed to result from binding of antigen-antibody complexes (10–12), has more recently been attributed to direct interaction of nephritogenic antibody with fixed glomerular antigen(s) (8, 9), usually proposed to be a component of the GBM. Our localization of gp330 in coated pits at the base of the foot processes where they face the GBM, together with our observation that anti-gp330 antibodies given intravenously to normal rats (passive HN) are found in identical coated pits in the same location, raises the exciting possibility that the coated pits on the "soles" of the foot processes may represent the sites where the circulating nephritogenic, anti-gp330 antibody and the membrane-associated gp330 antigen meet. The subepithelial location of the immune complexes could be explained if such complexes are subsequently shed into the lamina rara externa and only inefficiently or slowly removed.

Summary

The nephritogenic antigen of Heymann's nephritis (HN) was previously purified from tubular brush-border fractions of rat kidney and found to be a 330,000-mol-wt glycoprotein (gp330). This study was conducted to determine whether gp330 is also present in the rat glomerulus, and, if so, to establish where in the glomerulus it is located. Rabbit polyclonal and mouse monoclonal antibodies were raised against purified gp330, which specifically immunoprecipitated gp330 from solubilized brush-border fractions and specifically stained microvilli and coated invaginations (located at the base of the microvilli) of proximal tubule cells. Accordingly, they were used to localize gp330 by immunoprecipitation and immunocytochemistry in glomeruli of normal Lewis rats.

For immunoprecipitation, purified glomerular fractions were prepared from [^{35}S]-methionine-labeled kidneys, extracted with Triton X-100, and the extract was used for immunoprecipitation with affinity-purified rabbit polyclonal, or mouse monoclonal, anti-gp330 IgG. Analysis of immunoprecipitates by sodium dodecyl sulfate-polyacrylamide gel electrophoresis fluorography indicated that a band corresponding in mobility to gp330 was specifically precipitated.

When unfixed cryostat sections were incubated for indirect immunofluorescence with monoclonal or affinity-purified polyclonal IgG, a fine granular fluorescent staining was seen throughout the glomerulus. When aldehyde-fixed cryostat sections were incubated for indirect immunoperoxidase, reaction product was detected only in the epithelial cells and was not seen in the GBM, endothelium, or mesangium. Within the epithelium it was localized to the endoplasmic reticulum, occasional Golgi elements, multivesicular bodies, and coated pits at the cell surface. The reactive coated pits were distributed all along the cell membrane, including the sides and base of the foot processes. Reaction product was detected in the latter location only in sections that had been digested with neuraminidase before antibody incubation.

When rats were given rabbit anti-gp330 IgG by intravenous injection and their kidneys stained for direct immunoperoxidase 3 d later, rabbit IgG was seen to be deposited beneath the slit diaphragms and in the coated pits at the base of the foot processes.

The immunocytochemical and immunoprecipitation data indicate, in confirmation of the results of others, that the nephritogenic HN antigen is present in renal glomeruli

as well as in proximal tubular brush borders. The immunocytochemical results further demonstrate that gp330 is an epithelial, rather than a glomerular basement membrane, antigen. It appears to be synthesized by glomerular epithelial cells and subsequently becomes concentrated in coated pits. As both the endogenous antigen (gp330) and exogenously administered anti-gp330 antibody were localized to coated pits, it seems likely that coated pits located at the base of the foot processes are the sites where the HN antigen (gp330) and circulating antibodies directed against gp330 meet and where immune complexes are formed.

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