

# Microdomains of Distinctive Glycoprotein Composition in the Kidney Proximal Tubule Brush Border

DONTSCHO KERJASCHKI, LALITA NORONHA-BLOB,\* BERTRAM SACKTOR,\* and MARILYN GIST FARQUHAR

*Department of Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06460; and*

*\*Gerontology Research Center, National Institute of Aging, National Institutes of Health, Baltimore, Maryland 21224*

**ABSTRACT** Two membrane proteins, maltase and gp330 (the pathogenic antigen of Heymann nephritis), present in the proximal tubule brush border have recently been independently purified and found to be large glycoproteins of similar molecular weight ( $M_r = \sim 300,000$ ) by SDS PAGE. To determine the relationship between the two, monoclonal antibodies raised against the purified proteins were used for comparative immunochemical analyses and immunocytochemical localization. When a detergent extract of [ $^{35}\text{S}$ ]methionine-labeled rat renal cortex was used for immunoprecipitation with monoclonal antimaltase IgG, a single band of  $\sim 300$  kdaltons was precipitated, whereas a single 330-kdalton band was precipitated with monoclonal anti-gp330 IgG. Monoclonal antimaltase (gp300) IgG also immunoprecipitated maltase activity from solubilized renal maltase preparations, whereas monoclonal anti-gp330 IgG failed to do so. When cyanogen bromide-generated peptide maps of the two proteins were compared, there were many similar peptides, but some differences. When maltase and gp330 were localized by indirect immunofluorescence and by indirect immunoperoxidase and immunogold techniques at the electron microscope level, they were found to be differently distributed in the brush border of the initial ( $S_1$  and  $S_2$ ) segments of the proximal tubule: maltase was concentrated ( $\sim 90\%$ ) on the microvilli, and gp330 was concentrated ( $\sim 90\%$ ) in the clathrin-coated apical invaginations located at the base of the microvilli. We conclude that maltase (gp300) and the Heymann nephritis antigen (gp330) are structurally related membrane glycoproteins with a distinctive distribution in the proximal tubule brush border which may serve as markers for the microvillar and coated microdomains, respectively, of the apical plasmalemma.

The luminal aspect of the kidney proximal tubule consists of a brush border that is differentiated into two distinct types of structures—microvilli and intermicrovillar apical invaginations (1). The microvilli appear to closely resemble those of the intestine in their absorptive function and supporting cytoskeletal elements (2, 3). The apical invaginations are known to function in endocytosis (1) and have recently been shown to have extensive clathrin coats on their cytoplasmic surface (4), thus resembling clathrin-coated pits in other locations. At present there is relatively little information available on the comparative protein composition of these two proximal tubule structures. Microvillar fractions (which consist mainly of vesicles derived from microvilli along with some

contaminating intermicrovillar membranes), have been isolated and analyzed (5) and found to contain numerous proteins, some of which are cytoskeletal and others are transmembrane glycoproteins identified as hydrolytic enzymes (aminopeptidases, disaccharidases, etc.) (5) or transport systems (e.g., for glucose and amino acids) (6). Evidence has been presented that the protein composition of isolated microvilli differs from that of basolateral membrane subfractions (7). There is also suggestive evidence (8) (based on SDS gel analysis of enriched fractions) that the protein composition of the apical pinocytic invaginations differs from that of the microvillar and basolateral membranes, but there is no information available on the distribution of individual membrane proteins

in these plasmalemmal domains.

We have recently isolated and purified, from rat proximal tubule microvillar fractions, a large ( $M_r = 330,000$ ) membrane glycoprotein, gp330, of unknown function which constitutes the pathogenic antigen of Heymann nephritis, an experimental glomerulonephritis in rats (9). In a separate study, the enzyme maltase ( $\alpha$ -D-glucoside glucohydrolase), the major disaccharidase present in the proximal tubule membrane, was isolated and purified from the rat kidney (10) and found to be similar in size (based on SDS gel analysis) to gp330. To clarify the relationship between these two membrane constituents, we undertook a comparative immunochemical and immunocytochemical analysis of their properties, and we have found that they are closely related proteins that, interestingly, are located in different microdomains of the proximal tubule brush border. The results thus provide evidence that the intermicrovillar and microvillar plasmalemmal microdomains differ in their membrane glycoprotein composition.

## MATERIALS AND METHODS

### Animals

Male Sprague-Dawley or Lewis rats (200–250 g) obtained from Charles River Laboratories (Wilmington, MA) were used in these experiments. Female Wistar-derived rats (6 mo old) from the Animal Facility, Gerontology Research Center, National Institute on Aging, were the source of the maltase used in the enzyme assay.

### Materials

Glutaraldehyde-activated silica beads were purchased from Boehringer GmGH (Mannheim, Federal Republic of Germany); DEAE-BioGel A, BioGel A-5m (100–200 mesh), acrylamide, bisacrylamide, high molecular weight standards for SDS PAGE, and 2- $\beta$ -mercaptoethanol were from Bio-Rad Laboratories (Richmond, CA). Protein A-Sepharose 4B, cyanogen bromide (CNBr) activated-Sepharose 4B, lentil-lectin Sepharose 4B, Sephacryl S-500 Superfine, fluorescein isothiocyanate (FITC)-Protein A, and Concanavalin A (Con A) were purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Cyanogen bromide, chloramine T, pepstatin A, antipain, benzamidine chloride,  $\alpha$ -methyl-D-mannoside, ovalbumin, and bovine hemoglobin (Type II) were obtained from Sigma Chemical Co. (St. Louis, MO). [ $^{35}$ S]Methionine (specific activity >600 Ci/mM) and Na $^{125}$ I was purchased from Amersham Corp. (Arlington Heights, IL), and En $^3$ Hance and Triton X-100 were from New England Nuclear (Boston, MA). Diisopropyl-fluorophosphate was obtained from Aldrich Chemical Co. (Milwaukee, WI), and sodium deoxycholate from Calbiochem-Behring Corp. (La Jolla, CA). Rabbit-anti mouse IgG was purchased from DAKO (Accurate Chemical & Scientific Corp., Westbury, NY) or from Sigma Chemical Co. F(ab') $_2$  fragments of goat-anti mouse IgG conjugated to fluorescein and FITC-conjugated rabbit anti-goat IgG were from Cappel Laboratories, Inc. (Cochranville, PA), F(ab) fragments of sheep anti-mouse IgG conjugated to horseradish peroxidase from the Institute Pasteur Productions (Marnes La Coquette, France), and protein A conjugated to gold particles (10–15 nm diam) from E-Y Laboratories (San Mateo, CA). Glutaraldehyde was purchased from E. M. Sciences (Fort Washington, PA). Eagle's minimum essential medium (MEM) with Dulbecco's salts was from Gibco Laboratories, Inc. (Grand Island, NY). XO-Mat RP-5 x-ray film was obtained from Eastman-Kodak Co. (Rochester, NY). Cronex intensifying screens from DuPont Instruments (Wilmington, DE), and nitrocellulose paper (0.45  $\mu$ m) from Schleicher and Schuell, Inc. (Keene, NH).

### Experimental Procedures

**SOURCES OF ANTIBODIES:** The methods for the purification of gp330 (9) and maltase (10) used as antigens are given in detail elsewhere. In brief, gp330 was purified from detergent-solubilized, renal microvillar fractions (11)

by gel filtration (on BioGel A-5m) and lentil lectin affinity chromatography. Maltase was isolated from papain digests of homogenates of rat kidney cortex by ammonium sulfate precipitation, gel filtration, and affinity chromatography on a Tris-coupled Sepharose CL-6B column (10). Maltase activity was determined by the method of Berger and Sacktor (12).

Polyclonal anti-gp330 IgG was obtained from a rabbit immunized against purified gp330 as described previously (13). IgG was purified on Protein A-Sepharose 4B.

Monoclonal antibodies directed against gp330 (the Heymann nephritis antigen) were obtained from clone D155F21 (IgG2 subclass) (13) and those for active maltase from clone 1F12G1 (IgG $_1$  subclass). IgG was purified from ascites by ammonium sulfate precipitation followed by ion-exchange chromatography on DEAE-BioGel A (15) or by affinity chromatography on a Protein A-Sepharose 4B column (14).

**BIOSYNTHETIC RADIOLABELING OF RAT KIDNEYS:** Isolated rat kidneys were perfused for 4 h with MEM, containing 2 mCi [ $^{35}$ S]methionine in a perfusion apparatus (13). The cortices were extracted in RIPA buffer (16) (0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl buffer, pH 7.2) containing protease inhibitors (1 mM diisopropyl-fluorophosphate, 1  $\mu$ g/ml antipain, 1  $\mu$ g/ml pepstatin A, 1 mM benzamidine) and centrifuged at 40,000  $g$  for 30 min, and the supernatant was stored at  $-20^\circ\text{C}$  until used for immunoprecipitation.

**IMMUNOPRECIPITATION:** [ $^{35}$ S]Methionine-labeled cortical extract ( $\sim 100,000$  cpm) was incubated with anti-gp330 or anti-maltase IgG ( $\sim 20$   $\mu$ g) for 1 h at  $20^\circ\text{C}$ . With monoclonal IgGs, rabbit anti-mouse IgG ( $\sim 75$   $\mu$ g) was used as second antibody, and the resultant immune complexes were bound to protein-A-Sepharose 4B, as detailed previously (9), washed (three times) in RIPA-buffer, dissolved in 50  $\mu$ l, 2  $\times$  concentrated SDS PAGE sample buffer (final concentration = 3.65% SDS, 18 mM dithiothreitol, 4.4 mM EDTA, 10% glycerol) and analyzed by SDS PAGE.

**SDS PAGE:** Samples obtained as just described were analyzed on 3.6–8% acrylamide gradient gels in a Maizel-buffer system (17). Gels were fixed and stained in 50% methanol, 7% acetic acid, 0.2% Coomassie Blue R, destained in 25% methanol-7% acetic acid, soaked in En $^3$ Hance, dried, and exposed for fluorography on x-ray film at  $-70^\circ\text{C}$ .

**PROTEIN BLOTTING AND CON A OVERLAYS:** The SDS PAGE bands obtained by separation of kidney microvillus proteins were electrophoretically transferred to nitrocellulose paper (18) in 0.5% Tris buffer containing 0.25% boric acid at  $4^\circ\text{C}$  for 4 h at constant voltage (30 V). The resultant blot was quenched in hemoglobin buffer (0.1% bovine hemoglobin, 150 mM NaCl, 10 mM CaCl $_2$ , 10 mM MnCl $_2$ , in 25 mM Tris-HCl buffer, pH 7.2) at  $20^\circ\text{C}$  for 2 h, followed by incubation with  $3 \times 10^6$  cpm [ $^{125}$ I]-Con A (radiolabeled by the Chloramine T method [19]) in hemoglobin buffer for 1 h at  $4^\circ\text{C}$ . Transfers were washed in buffer until no further radioactivity was released and exposed for autoradiography. Control incubations and washes were carried out under the same conditions except that 250 mM  $\alpha$ -methyl-D-mannoside was included.

**PURIFICATION OF GP330 AND MALTASE ON MONOCLONAL ANTIBODY-SEPHAROSE 4B FOR PEPTIDE MAPPING:** Microvillar fractions were prepared (11) from Sprague-Dawley rat kidneys and extracted in 1% Triton X-100 in PBS containing protease inhibitors (given above) at  $20^\circ\text{C}$  for 20 min. [ $^{125}$ I]-Labeled microvillar extracts ( $\sim 10,000$  cpm) were added as tracer. The extracts were centrifuged at 40,000  $g$  for 20 min, and the supernatants were collected and incubated for 12 h at  $4^\circ\text{C}$  with 1 ml glutaraldehyde-activated silica beads, containing  $\sim 10$  mg monoclonal anti-gp330 IgG (from clone D $_{155F21}$ ). The supernatant from this incubation was subsequently incubated with silica beads conjugated with monoclonal antimaltase IgG (from clone 1F12G1), containing  $\sim 5$  mg IgG/ml, for 12 h at  $4^\circ\text{C}$ . The beads were then washed (10 times) in 1% Triton X-100 in PBS, until radioactivity was no longer detectable. The bound material was released by incubation in 6 M urea, 1% sodium deoxycholate, 25 mM Tris-HCl buffer, pH 8.6, at  $60^\circ\text{C}$  for 5 min. Samples were applied to a Sephacryl S-500 column (to remove traces of released IgG) and eluted with the urea-Tris-sodium deoxycholate buffer given above. Fractions containing the radioactivity (typically 250–500 cpm per fraction) were pooled and dialyzed (against a total of 20 liters of distilled water) for 4 d at  $4^\circ\text{C}$ . A bulky white precipitate formed of which an aliquot was analyzed for protein content (20), and the rest was collected by centrifugation in a Microfuge B (Beckman Instruments, Inc., Palo Alto, CA), lyophilized, and stored at  $-20^\circ\text{C}$ . Purity was assessed by SDS PAGE.

**PEPTIDE MAPPING:** Affinity-purified gp330 or maltase (150  $\mu$ g) was suspended in 20  $\mu$ l 70% formic acid in a 1.5-ml Eppendorf tube; 20  $\mu$ g cyanogen bromide was added (21), the vials were flushed with nitrogen, covered with aluminum foil, and incubated for 12 h at  $20^\circ\text{C}$ , after which 1  $\mu$ l of 2- $\beta$ -mercaptoethanol was added, and cleavage was continued for another 8 h at  $20^\circ\text{C}$ . The resultant protein fragments were dried with a mild stream of nitrogen, dissolved in 20  $\mu$ l sample buffer by boiling for 5 min, separated on a 10–20% gradient SDS gel, and fixed and stained as described above.

To visualize the glycopeptides, gels were destained, neutralized with Tris-

<sup>1</sup> Abbreviations used in this paper: Con A, concanavalin A; ER, endoplasmic reticulum; FITC, fluorescein isothiocyanate; kd, kilodaltons; PLP, paraformaldehyde-lysine-periodate.

buffered saline, soaked in hemoglobin buffer, and incubated in [<sup>125</sup>I]Con A (3 × 10<sup>6</sup> cpm) in hemoglobin buffer (22). The gels were subsequently washed in the same buffer until the radioactivity in the washes was at background, and then dried and exposed 4–8 h for autoradiography.

**IMMUNOPRECIPITATION OF MALTAZE ACTIVITY:** Purified maltase (0.25 U) prepared from female Wistar rats (23) was incubated overnight at 4°C with ~50 μg anti-maltase monoclonal IgG, or anti-gp330 monoclonal IgG in 200 μl of 0.01 M NaH<sub>2</sub>PO<sub>4</sub> buffer, pH 7.4. The following morning, 50 μg of rabbit anti-mouse IgG was added to the tube and the mixture was reincubated overnight at 4°C. The pH of the incubation medium was then adjusted (to 8.0) and the ionic strength was raised (to 0.1 M) by addition of 0.25 M Na H<sub>2</sub>PO<sub>4</sub>. Protein A-Sepharose 4B beads (0.2 ml of a 50% suspension in 0.1 M Na H<sub>2</sub>PO<sub>4</sub>, pH 8.0) were added and the mixture was rotated for 1 h at room temperature. The beads were pelleted by centrifugation, and the maltase activity remaining in the supernatant was measured. Controls consisted of incubations carried out without addition of IgG in which there was no depletion of maltase activity.

### Preparation of Tissues for Immunocytochemistry

Rat kidneys were flushed with MEM by perfusion via the abdominal aorta for 5 min at 4°C (24). Some kidneys were then fixed by perfusion with 4% formaldehyde (freshly prepared from paraformaldehyde), plus 0.1% glutaraldehyde in 100 mM cacodylate buffer, pH 7.2, for 3–5 min. This was followed by perfusion with 50 mM glycine in PBS (to quench free aldehyde groups) (24, 13). Alternatively, kidneys were perfused with the paraformaldehyde-lysine-periodate (PLP) fixative of McLean and Nakane (25) by perfusion for 10 min, followed by immersion of pieces of cortical tissue for a total of 4 h at 20°C. Tissues fixed by either method were soaked 1 h at 20°C in 10% DMSO in PBS, frozen in nitrogen-cooled isopentane (24, 13), and stored in liquid nitrogen until used for immunoperoxidase or immunogold localizations. Some PLP-fixed specimens were soaked in 1.2 M sucrose in PBS, frozen in liquid nitrogen, and used for preparation of 0.5-μm frozen sections (26).

**IMMUNOFLUORESCENCE:** 0.5-μm sections were prepared (26) from PLP-fixed tissue blocks on a Reichert Ultracut ultramicrotome equipped with a cryo-attachment. They were then quenched in 50 mM NH<sub>4</sub>Cl (30 min) and incubated with monoclonal anti-gp330 or antimaltase IgG (30 min), washed and incubated (30 min each) with FITC-goat anti-mouse F(ab')<sub>2</sub> (30 min) that had been depleted of cross-reactivity with rat IgG (13). All incubations were done at room temperature. Sections reacted with monoclonal antimaltase IgG were further incubated with FITC-rabbit anti-goat IgG and FITC-Protein A, in order to amplify the fluorescent signal. All sections were examined and photographed on a Zeiss Photomicroscope II equipped with phase-contrast optics and epifluorescence.

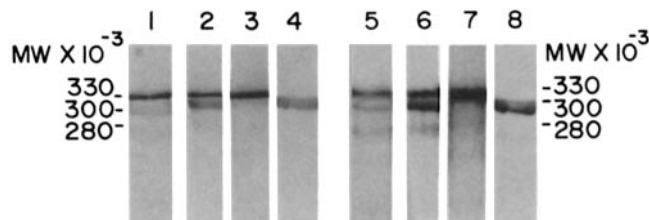
**IMMUNOPEROXIDASE:** Cryostat sections (~15 μm) prepared from kidneys fixed either in the formaldehyde-glutaraldehyde mixture or PLP were reacted according to methods detailed elsewhere (13, 24, 27). Briefly, sections were incubated in the monoclonal antibodies (~25 μg/ml) for 4–12 h, washed (4–6 h) in PBS-ovalbumin, and incubated in peroxidase-conjugated sheep anti-mouse F(ab) (1:50) for 2 h at 20°C, after which they were postfixed in 1.5% glutaraldehyde in 100 mM cacodylate buffer (1 h), washed again (3 h), and reacted in diaminobenzidine medium (28) for 2–10 min. Sections were then postfixed in OsO<sub>4</sub> reduced by the addition of ferrocyanide (29), dehydrated, and embedded in Epon 812. Ultrathin sections were stained with lead citrate and examined in a Philips 301 electron microscope.

**IMMUNOGOLD:** Cryostat sections were prepared from kidney tissue fixed and quenched as described above for immunoperoxidase. They were incubated in monoclonal IgG (~25 μg/ml) in PBS containing 0.1% ovalbumin for 12 h at 4°C. Sections were then washed (three times) in PBS-ovalbumin for 1 h at 20°C, and incubated for 2 h with rabbit anti-mouse IgG (~20 μg/ml). After washing (three times) for 1 h in PBS-ovalbumin, they were exposed to Protein A-gold complexes (diluted 1:4) for 12 h at 20°C, washed again (three times) in PBS-ovalbumin for 1 h, postfixed in 1.5% glutaraldehyde in cacodylate buffer, pH 7.4, for 45 min, and treated with veronal-acetate-buffered OsO<sub>4</sub> (45 min) and uranyl acetate (1 h) in the same buffer before dehydration. Ultrathin sections were doubly stained with uranyl acetate and lead citrate.

## RESULTS

### Characterization of Antibody Specificities by Immunoprecipitation

When [<sup>35</sup>S]methionine-labeled renal cortical extracts were immunoprecipitated with polyclonal IgG prepared from the serum of a rabbit immunized with electrophoretically pure gp330 (13), two heavy bands with an apparent *M<sub>r</sub>* of 330,000



**FIGURE 1** Electrophoretogram (3.6–8% gradient gel) of the immunoprecipitates obtained from [<sup>35</sup>S]methionine-labeled cortical extracts with anti-gp330 and antimaltase antibodies. Only the high molecular weight region of the gel is shown, either stained by Coomassie Blue (lanes 1–4), or in the corresponding fluorogram (lanes 5–8). Lanes 1 and 5 show the starting material which contains a prominent 330-kD band, a lighter 300-kD band, and a faint 280-kD band. The latter is visible primarily in the fluorogram. The immunoprecipitate obtained with rabbit anti-gp330 IgG (raised against electrophoretically pure gp330) contains all three bands (lanes 2 and 6); that obtained with monoclonal anti-gp330 contains only the 330-kD band (lanes 3 and 7); and that obtained with monoclonal antimaltase IgG contains only the 300-kD band (lanes 4 and 8).

and 300,000 (gp330) were precipitated (Fig. 1). These bands were readily visible in Coomassie Blue-stained gels (Fig. 1, lane 2) and in the corresponding fluorograms (Fig. 1, lane 6). In addition, a faint band with an apparent *M<sub>r</sub>* of ~280,000 was usually observed, especially in the fluorogram (Fig. 1, lane 6). Previously only one band was detected by immunoprecipitation (13) with the same antibody. The ability to resolve two to three reactive bands in these preparations can be attributed to improved resolution in the high molecular weight region of the gel.

When monoclonal IgG produced by clone D<sub>5</sub>F<sub>2</sub>1 (derived from a mouse immunized with purified gp330 [13]) was used, only the 330-kilodalton (kd) band (gp330) was precipitated (Fig. 1, lanes 3 and 7). When monoclonal IgG produced by clone 1F12G1 (derived from a mouse immunized with active maltase [14]) was used, only the 300-kD band was seen (Fig. 1, lanes 4 and 8). Thus, the 300-kD band corresponds to active maltase (14).<sup>2</sup>

### Immunoprecipitation of Maltase Activity by Anti-gp330, but Not Anti-gp 330 IgG

Monoclonal IgG produced by clone 1F12G1, which binds to the catalytically active form of purified maltase (gp300) (14), immunoprecipitated up to 95% of the maltase activity. In contrast, monoclonal anti-gp330 IgG failed to precipitate maltase activity. As might be anticipated, polyclonal rabbit anti-gp330 (which immunoprecipitates both gp330 and active maltase) partially depleted the supernatant of enzymatic activity (~60%), when tested at a moderate concentration.

### [<sup>125</sup>I]Con A Binds to Both Gp330 and Gp300 on Nitrocellulose Overlays

[<sup>125</sup>I]Con A bound to a number of the bands transferred from gels of isolated microvilli (Fig. 2a), including gp330 and gp300. The specificity of the lectin binding was demonstrated

<sup>2</sup> The discrepancy between the *M<sub>r</sub>* (~300,000) of maltase as detected in this study and that (~330,000) determined for maltase in a previous paper (10) may be due to the variability in the migratory behavior of this large glycoprotein in the different SDS PAGE systems.

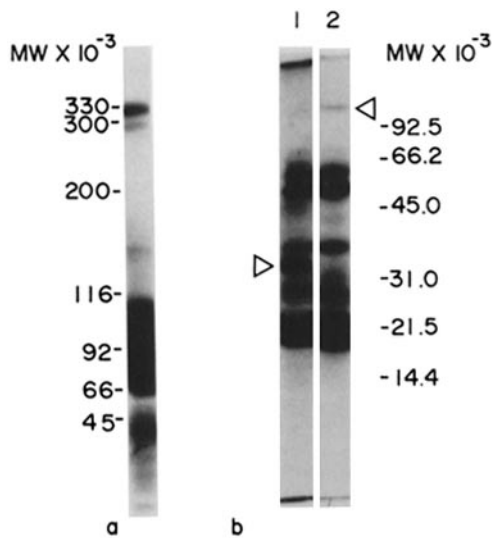


FIGURE 2 (a) Con A overlay of a nitrocellulose transfer of a microvillar fraction, showing that intact gp330 and gp300 as well as a number of other membrane components bind Con A. (b) Cyanogen bromide peptide maps obtained from affinity-purified gp300 (lane 1) and gp330 (lane 2) after direct overlaying of an SDS gel with [ $^{125}$ I]Con A as described in the Materials and Methods. The glycopeptides derived from these proteins show extensive homologies, e.g., at ~50, 25, and 19 kd. Peptides not shared are a heavily glycosylated fragment with an apparent  $M_r$  of 31,000 in gp300 (arrowhead, lane 1) and a light band with an apparent  $M_r$  of 100,000 in gp330 (arrowhead, lane 2).

by the fact that complete inhibition of labeling was obtained in the presence of 250 mM  $\alpha$ -methyl mannoside. Thus, both gp300 and gp330 are glycoproteins.

**GLYCOPEPTIDE MAPS FROM PURIFIED GP330 AND GP300:** Comparison of the glycopeptides generated from affinity-purified (on monoclonal antibody columns) gp330 and maltase revealed major similarities and some differences (Fig. 2b): six of the major [ $^{125}$ I]Con A-labeled bands appeared identical (compare lanes 1 and 2), but a major glycopeptide with an apparent  $M_r$  of ~31,000 was observed on the gp300 (lane 1) but not in the gp330 (lane 2) fragments, and a lightly labeled band of an apparent  $M_r$  of ~100,000 was seen in the gp330 cleavage products, but not in the maltase cleavage products. These data indicate that gp330 and active maltase possess a substantial amount of structural homology, but they are not identical. The fact that gp330 and gp300 show extensive structural homology could explain why a polyclonal anti-gp330 that recognizes both proteins was produced by immunization with chromatographically pure gp330.

### Immunocytochemical Localization of Gp330 and Maltase (gp300) in the Proximal Tubule

**LIGHT MICROSCOPY:** By immunofluorescence on 0.5- $\mu$ m frozen sections, it was apparent that the patterns of brush border staining obtained with monoclonal antibodies to gp330 and to maltase differed: Anti-gp300 (maltase) was located in the region corresponding to that of the microvilli (Fig. 3, a and b), whereas staining for gp330 was concentrated in a band at the base of the microvilli (Fig. 3, c and d) in the region where the apical invaginations are known to be located. This difference in staining pattern was even more striking in 1- $\mu$ m plastic sections of specimens incubated for immunoperoxidase (Figs. 4 and 5). Rabbit anti-gp330 (which recognizes both

gp330 and gp300) stained both sites evenly (not shown).

**IMMUNOPEROXIDASE:** Examination by electron microscopy of specimens reacted by the indirect immunoperoxidase procedure (Figs. 6–9) confirmed the differential localization of maltase and gp330. Reaction product for maltase was confined almost exclusively to the microvilli where it was located on the outer aspect of the microvillar membranes (Fig. 6). Relatively few apical invaginations or coated vesicles were labeled. Moreover, the distribution of maltase was identical in all tubular segments.

By contrast, in optimally reacted specimens,<sup>3</sup> reaction product for gp330 was heavily concentrated in the clathrin-coated, apical invaginations found at the base of the microvilli (Figs. 7 and 8). The restricted distribution of gp330 was characteristic for the S1 and S2 segments of the proximal tubules; in S3 segments, where apical invaginations are known to be less abundant (1), reaction product was also present on the microvillar membranes.

The intracellular distribution of gp300 and gp330 also differed: reaction product for gp300 (maltase) was restricted to a few lysosomes (Fig. 4). However, reaction product for gp330 was found in large apical vacuoles, presumably corresponding to endosomes (Fig. 9), some of which were surrounded by budding vesicles which also were reactive. In addition, in PLP-fixed preparations, reaction product was sometimes found in the rough endoplasmic reticulum (ER) and some Golgi-associated vesicles of tubular cells. Both gp330 and gp300 were consistently absent from the apical tubules (1) present in the apical cytoplasm (Figs. 6–9).

**IMMUNOGOLD:** When protein A-gold was used as the second reagent (Figs. 10–13), it bound to the exposed surface of the proximal tubule cell to which it presumably had direct access by diffusion through the open lumina; however, it did not penetrate the cells and bind to intracellular structures. Apparently, diffusion of this large probe into intracellular organelles is quite limited under the conditions utilized. As far as its localization on surface structures is concerned, the results confirmed those obtained by immunoperoxidase: with the monoclonal reagents, maltase is concentrated on the microvilli (Fig. 10), whereas gp330 is concentrated in coated invaginations (Figs. 11 and 12). An advantage of the protein A-gold is that the reactivity can be quantitated by counting the gold particles. Counts of particles over the microvilli or coated apical invaginations (Table I) indicated that with monoclonal anti-gp330 IgG, ~86% of the particles were located over coated apical invaginations and only ~14% over microvilli, whereas with monoclonal anti-maltase, ~95% of the particles were associated with microvilli. With rabbit anti-gp330, which recognizes both gp330 and maltase by immunoprecipitation, gold particles were more evenly distributed on both microvilli and coated apical invaginations (Fig. 13).

### Presence of gp330 and Absence of Maltase (gp300) in Glomeruli

As reported previously (13), when gp330 is localized in the glomerulus by immunoperoxidase with either polyclonal or monoclonal anti-gp330 IgG, it is found exclusively in glomerular epithelial cells. In this cell type, as in the proximal tubule cell, it is also concentrated in coated pits, but the latter

<sup>3</sup> If specimens are over-reacted, e.g., when the diaminobenzidine reaction is prolonged, excess reaction product is generated which can diffuse and relocate onto adjacent structures (30).

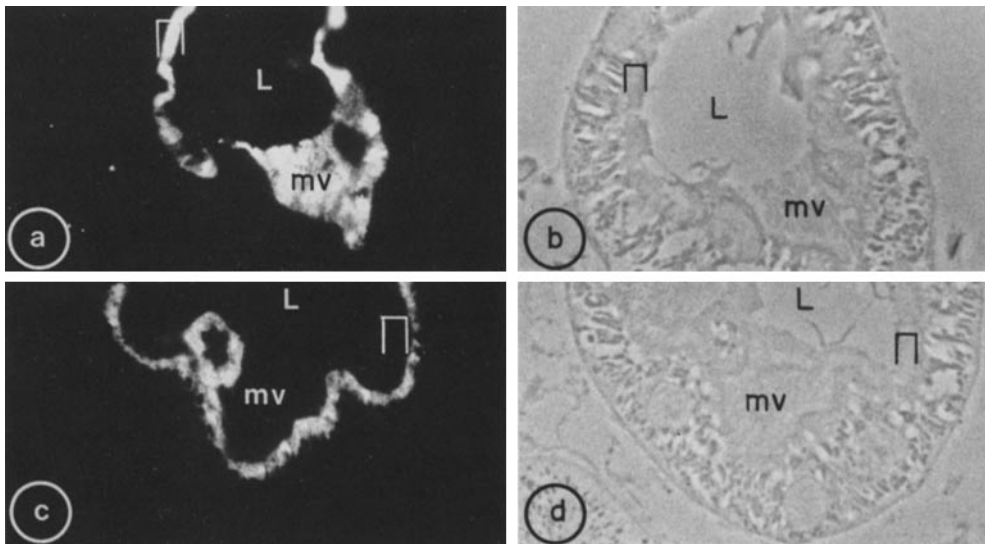
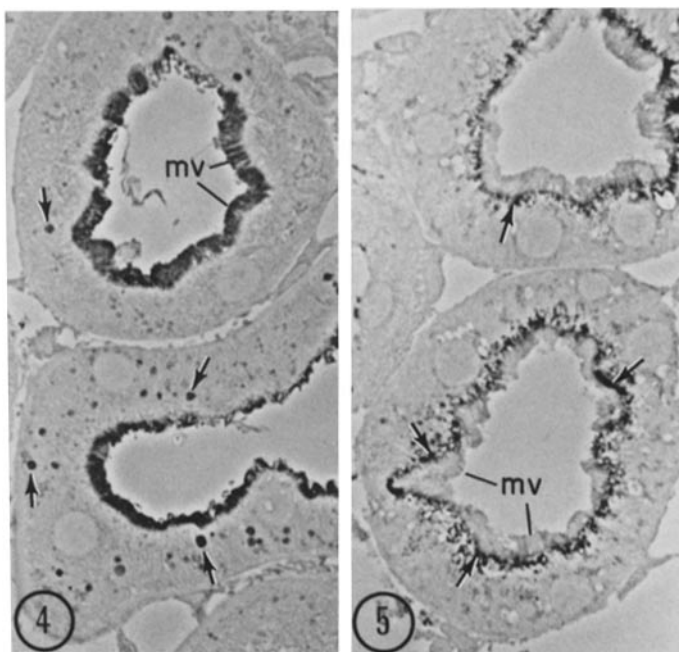


FIGURE 3 Indirect immunofluorescence (left) and corresponding phase-contrast micrographs (right) of 0.5- $\mu$ m frozen sections of a PLP-fixed rat kidney incubated either with monoclonal antimaltase IgG (a and b) or anti-gp330 (c and d) IgG. By comparing the two types of preparations it can be determined that maltase (a) is localized to a region corresponding to that of the brush border microvilli seen here cut both in normal section (brackets) and in a grazing section (mv). By contrast, gp330 (c) is localized in a band beneath the microvilli (brackets and mv) in a region corresponding to that of the apical invaginations. L, tubule lumen.  $\times$  675.



FIGURES 4 and 5 Indirect immunoperoxidase staining of 1- $\mu$ m plastic sections from a PLP-fixed rat kidney incubated with monoclonal antimaltase IgG (Fig. 4) or monoclonal anti-gp330 IgG (Fig. 5). Maltase (gp300) is localized to the proximal tubule microvilli (mv). It is also seen in some lysosomal structures in the cytoplasm (arrows). Gp330 is concentrated in a zone (arrows) located at the base of the microvilli.  $\times$  450.

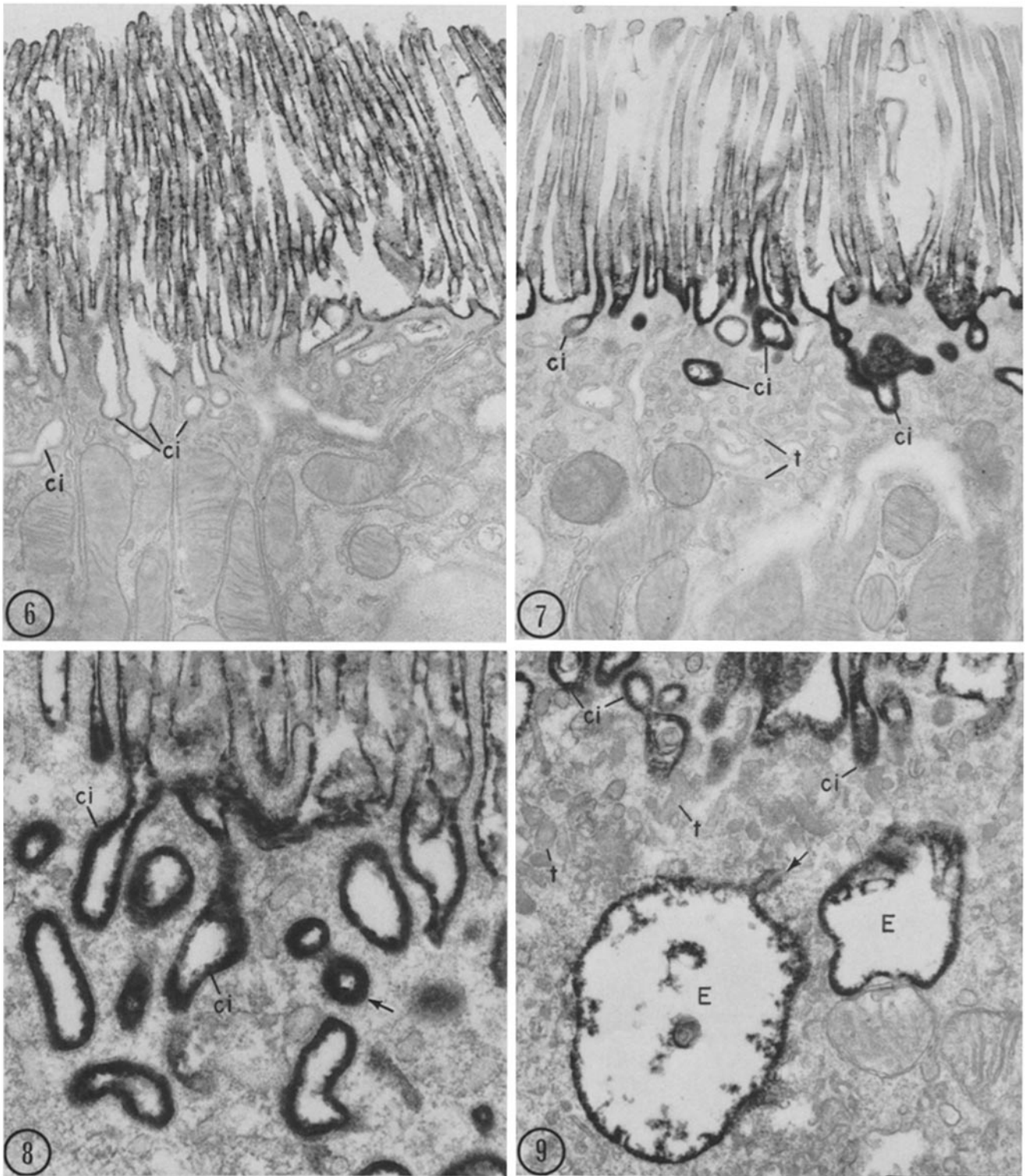
are distributed over the entire cell surface, i.e., along the cell bodies and both the sides and the base of the foot processes. In addition, by immunoperoxidase, the antigen can be regularly detected intracellularly in some ER and Golgi cisternae and in multivesicular bodies. Its location in ER and Golgi cisternae together with biosynthetic data on isolated glomeruli (13) indicate it is synthesized by the glomerular epithelium. With the immunogold technique, the antigen can be detected in coated pits along those regions of the epithelial cell surface that are exposed to the urinary spaces and therefore accessible (by diffusion) to the protein A-gold reagent (Fig. 14). By contrast, when monoclonal antimaltase IgG was used as the first antibody, no binding to any glomerular structures was detected by either the immunoperoxidase or immunogold techniques.

#### DISCUSSION

We have shown that two structurally related but different glycoproteins, gp300 (renal maltase [14]) and gp330 (the

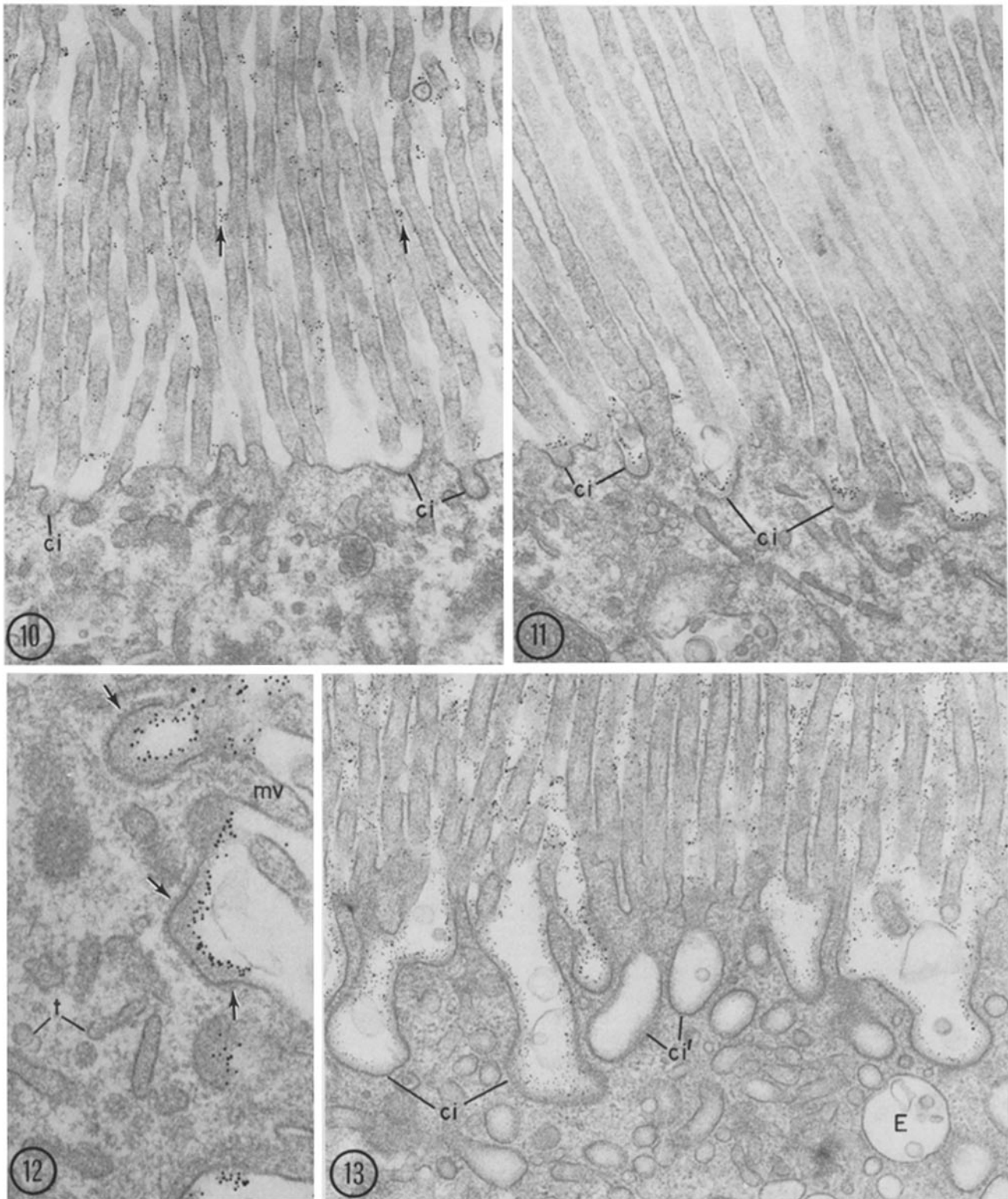
pathogenic antigen of Heymann's nephritis [9]), have distinctive distributions on the luminal membrane of the proximal kidney tubule: maltase is concentrated in the microvilli whereas gp330 is concentrated in the clathrin-coated (4), apical invaginations.

It is well known that the apical (microvilli) and basolateral domains of the proximal tubule (7) and other cells differ in their protein composition, and, given the expected fluidity of the bilayer, it is assumed that these differences are maintained by the junctional complexes because destruction of the junctions leads to mixing (31, 32). In this study we have defined two distinct microdomains, the microvillar and intermicrovillar microdomains, of different composition within the apical membrane itself. There are only a few instances where such microdomains of different composition have been detected: namely, in the luminal membrane of the vascular endothelium (33, 34), and in coated pits on various cell surfaces (35). In the case of the latter, some membrane constituents such as low-density lipoprotein, peptide hor-



FIGURES 6-9 Immunoperoxidase localization of maltase (Fig. 6) and gp330 (Figs. 7-9) in the brush border region of the proximal tubule. Maltase (gp300) is localized largely to the membranes of the microvilli (Fig. 6); the coated invaginations (*ci*) are unstained. Staining for gp330 is localized at the base of the microvilli (Fig. 7) where it is concentrated in coated apical invaginations (*ci*) (Figs. 7 and 8). Apical tubular structures (*t*) are not reactive for gp330. Fig. 8 shows a higher magnification view of a group of coated invaginations containing gp330, several of which (*ci*) are clearly in continuity with the apical cell membrane. Distinct cage-like (clathrin) coats are visible on some of them (arrow). Fig. 9 shows localization of gp330 in two endosomes (*E*) found just beneath the coated invaginations (*ci*). The membrane of a vesicle (arrow) which is also reactive for gp330 is continuous with the endosomal membrane. Note that abundant apical tubules (*t*) are present in the field, but they are not reactive. Figs. 6 and 7,  $\times 32,000$ ; Fig. 8,  $\times 66,000$ ; Fig. 9,  $\times 42,000$ .





FIGURES 10-13 Localization of maltase (Fig. 10) and gp330 (Figs. 11 and 12), as seen by labeling with protein A-gold. Fig. 10, which was incubated with monoclonal anti-maltase IgG, shows small clusters of gold particles concentrated on the microvilli (arrows), whereas the intermicrovillar coated invaginations (*ci*) are not labeled. The distribution of gold in Figs. 11 and 12, incubated with monoclonal anti-gp330 IgG, is exactly reversed: gold particles are concentrated in coated invaginations (*ci*), whereas few bind to the microvilli. Fig. 12 is an enlargement of the apical cell membrane showing heavy binding of protein A-gold to coated invaginations on which clathrin-like coats are visible (arrows). The gold particles are separated from the cell membranes by a thick grey layer, presumably constituted by immunoglobulins plus the membrane proteins. Apical tubular structures (*t*) are devoid of label. Fig. 13 shows a field from a preparation incubated with polyclonal anti-gp330 IgG. As might be expected (since the antibody recognizes both gp330 and maltase by immunoprecipitation), protein A-gold binds to both the microvilli and some of the coated invaginations (*ci*). Several of the coated invaginations (*ci'*) and an endosomal vacuole (*E*) are devoid of gold, presumably owing to the inability of gold to diffuse into these structures. Figs. 10, 11, and 13,  $\times 42,000$ ; Fig. 12,  $\times 72,000$ .

TABLE I  
Counts of Protein A-Gold Particles on Brush Border  
Microdomains\*

Antibody	Location of protein A-gold particles	
	Microvilli	Intermicrovillar coated apical invaginations
Monoclonal anti-Gp330 (clone D <sub>1</sub> 55F <sub>2</sub> 1)	266 (13.8) <sup>‡</sup>	1,734 (86.2)
Monoclonal Antimaltase (clone 1F12G1)	1,892 (94.6)	108 (5.4)
Rabbit anti-gp330 <sup>§</sup>	1,410 (70.5)	590 (29.5)

\* Cryostat sections were incubated with a specific monoclonal anti-gp330, antimaltase, or polyclonal rabbit antibody followed by protein A-gold. Counts of gold particles were made on electron micrographs enlarged to  $\times 35,000$ . A minimum of 2,000 gold particles was counted per group on micrographs from three different proximal tubules. Only S<sub>1</sub> or S<sub>2</sub> segments were counted.

<sup>‡</sup> Numbers in parentheses indicate percent of the total particles counted.

<sup>§</sup> Recognizes both gp330 and maltase (by immunoprecipitation).

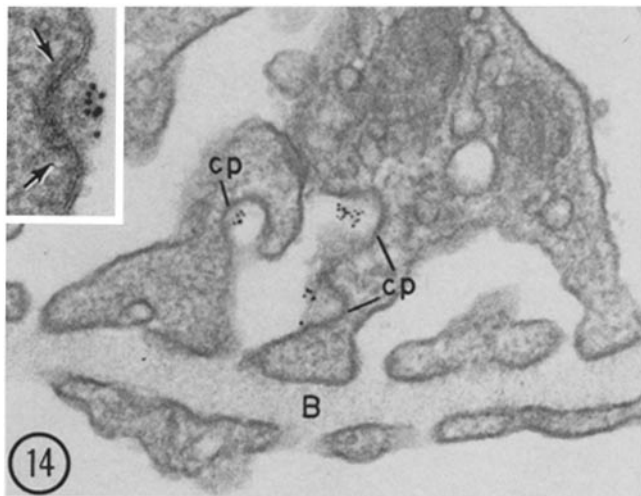


FIGURE 14 Immunogold localization of gp330 to several coated pits (cp) on glomerular epithelial cells. Clusters of protein A-gold are found in three coated pits located on the sides of the foot processes. The inset is a higher magnification view of another coated pit (delineated by arrows) containing a cluster of gold particles. B, basement membrane.  $\times 35,000$ ; inset,  $\times 89,000$ .

none, transferrin, and asialoglycoprotein receptors, recently referred to as "migratory" membrane constituents (36), are concentrated (or become concentrated upon ligand binding) in coated pits and vesicles. Others, such as the theta and H63 antigens (37, 38), referred to as "sessile" membrane constituents, are excluded from coated pits. It is the clathrin coats of the latter that are believed to stabilize the membrane, preventing mixing, and to thereby maintain the distinctive character of the noncoated and coated microdomains. In the renal brush border, the intermicrovillar domain is backed by a continuous clathrin coat (4), and the microvillar domain is backed by actin and other cytoskeletal proteins (3). Both families of proteins might be expected to play a role in maintenance of microdomain specificity.

It is reasonable to expect that besides gp330, gp300, and clathrin, other brush border membrane constituents may be differentially distributed in the microdomains of the renal brush border. Microvillar fractions are well known to contain several transport systems since they actively transport glucose and amino acids (6) *in vitro*. Upon SDS gel analysis they contain multiple protein bands only a few of which have been identified (3, 5). The clathrin-coated apical invaginations in the proximal tubule are known to function in endocytosis as in other systems. In fact the endocytic apparatus is highly amplified to facilitate reabsorption by the proximal tubule of proteins filtered by the glomerulus.<sup>4</sup> The bulk of the reabsorption occurs in the initial (S<sub>1</sub>) segments of the proximal tubule (41) where the endocytic apparatus is most highly developed, and this activity declines as one goes down the nephron (S<sub>2</sub> and S<sub>3</sub> segments) where the endocytic apparatus is less extensive and, interestingly, the differential segregation of gp330 into the apical invaginations is less complete.

The intracellular route taken by reabsorbed proteins in the proximal kidney tubule has been charted using a variety of tracers (1, 28, 42) and is similar to that in other systems: proteins are absorbed via apical invaginations which fuse with endosomes which in turn fuse with lysosomes. Besides the usual structures characteristic of the endosomal-lysosomal pathway, numerous, "apical tubules" (1) are present in the proximal tubule cell. Evidence has recently been presented by Christensen (43) that recycling of endocytosed membrane is accomplished via these structures. Interestingly, gp330 has been found in other elements along the endocytic pathway but was not detected in the apical tubules.

Previously, Bode and co-workers (8, 44) have isolated (by free-flow electrophoresis) microvillar fractions and fractions enriched either in pinocytotic vesicles, lysosomes, or basolateral membranes. They have detected clear differences (by SDS PAGE) in the protein composition of these fractions. However, with the exception of the microvilli, the overall distribution of various cellular membranes in these subfractions is not clear, and some types of structures (e.g., endosomes, apical tubules) were not identified in the subfractions.

It is intriguing that although gp330 and maltase (gp300) have a differential distribution in the brush border membrane, our data indicate that they are structurally related proteins as demonstrated by the fact that the majority of the glycopeptides generated by cyanogen bromide match. From the evidence available it cannot be determined whether these two proteins are products of separate genes or members of the same gene family or whether one arises from the other by co- and/or posttranslational modifications. Biosynthetic studies now in progress should help to clarify their relationship to one another.

The normal physiological functions of maltase and gp330 in the proximal tubule are unknown at present. A disaccharidase similar to renal maltase appears to be present in the intestinal brush border (45) where appropriate substrates (maltose and other glucose-terminating carbohydrate moieties) are present. In the kidney, maltose would not be expected to be

<sup>4</sup> In glomeruli of normal individuals, anionic molecules of a size  $<20$  Å are freely filtered and therefore present in the tubule lumen in the same concentration as in the plasma; intermediate-sized molecules ( $\sim 20$ – $70$  Å) encounter increasing restriction to filtration with increasing size up to  $70$  Å (the size of albumin) and are therefore present in the filtrate in decreasing amounts according to their size; molecules  $>70$  Å are present in only trace amounts (40).



present in significant amounts, and the natural substrates for this enzyme are unknown.

gp330 has been detected in the glomerular epithelium (13), as well as in the proximal tubule epithelium, where it is also a component of coated pits. Although its function is unknown, its presence in this location has ramifications for the pathogenesis of immune complex diseases of glomeruli. When rats are immunized against gp330, circulating anti-gp330 antibodies bind to the antigen located in coated pits at the base of the foot processes, and the resultant immune complexes accumulate in the lamina rara externa of the glomerular basement membrane and damage it (13).

In an attempt to gain insight into the distribution and thereby the function of gp330, we have recently surveyed several tissues (46) and have found that this protein is a widely distributed but not a universal component of coated pits. It appears to be concentrated in coated pits in actively absorptive epithelia of the rat because we have detected it in the epithelium of the epididymis, and embryonic visceral yolk sac. In these tissues it is located in the same group of organelles as in kidney epithelia, i.e., in coated pits and endosomes or lysosomes (multivesicular bodies), as well as in rough ER and Golgi elements. The presence of this protein in coated pits suggests that it is somehow involved, as receptor or ligand, in receptor mediated events. The localization of the protein in biosynthetic compartments (ER and Golgi) suggests that all cell types mentioned synthesize gp330. Thus, we favor the idea that gp330 is either a constitutive component of coated pits or a receptor present in some coated pits for which the ligand (or ligands) is unknown.

In summary, we have obtained evidence that the microvillar and intermicrovillar plasmalemmal microdomains of the proximal tubule brush border differ in their protein composition, and we have provided evidence that gp300 and gp330, respectively, represent markers for these two microdomains. In the future, it will be of interest to isolate and analyze these two microdomains, in order to determine how extensively their composition differs.

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