The Membrane Composition of Coated Pits, Microvilli, Endosomes, and Lysosomes Is Distinctive in the Rat Kidney Proximal Tubule Cell

Jane Somsel Rodman, Lisa Seidman, and Marilyn Gist Farquhar

Department of Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06510

Abstract. The distribution of a number of membrane proteins on plasmalemmal microdomains (microvilli, coated pits) and in endosomes and lysosomes of the proximal tubule epithelial cell was determined in normal rat kidneys by immunofluorescence and immunoelectron microscopy. Two major brush border proteins, 130 and 94 kD, and γ-glutamyl transpeptidase were detected on the membranes of the microvilli but were not found on membranes of coated pits. Gp330, the Heymann nephritis antigen, and clathrin were localized in coated pits. The lysosomal membrane glycoprotein, lgp120 (Lewis, V., S. A. Green, M. Marsh, P. Vihko, A. Helenius, and I. Mellman, 1985, *J. Cell Biol.*, 100:1839–1847) was restricted to lysosomes where it co-localized with β-glucuronidase. Endosomes, identified by preloading with HRP injected 5–15 min before rats were killed, did not contain detectable amounts of any antigen tested. The distribution of the same proteins was also determined in rats given sodium maleate, which is known to slow or reduce protein absorption by the proximal tubule and to cause vacuolation of the endocytic apparatus. After maleate treatment the distribution of microvillar and lysosomal markers was unchanged, but the coated pit markers were redistributed—gp330 was concentrated in newly formed apical vacuoles, and clathrin was diffusely distributed in the apical cytoplasm or on apical coated vesicles. These findings indicate that the membrane composition of microvilli, coated pits, endosomes, and lysosomes is distinctive in the proximal tubule cell; and that gp330, unlike other known coated pit membrane components, is not transferred to endosomes during endocytosis. After maleate treatment, the coated pits lose their clathrin coats, and the corresponding membrane is internalized.

THE epithelium of the proximal kidney tubule is highly specialized for protein reabsorption by endocytosis (30, 42), and the morphology of the structures involved, i.e., the plasmalemma, coated pits, endosomes and lysosomes has been studied in some detail (8, 9, 12, 16, 29, 30, 42). Little is known, however, about the comparative composition of the membranes of these structures. Previous work from this laboratory has established that the apical plasmalemma along the proximal tubule brush border is differentiated into two structurally and functionally distinct microdomains, constituted by the microvilli and an extensive system of clathrin coated pits located at the base of the microvilli (23, 37). The coated pits are the sites of protein absorption, and their composition has been shown to differ from that of the microvilli: gp330, a membrane protein of unknown function is concentrated on the clathrin-coated microdomains whereas maltase, a structurally related glycoprotein, is concentrated on the microvilli (23). So far these two proteins are the only known markers for the two microdomains, and the extent to which the membrane composition of microvilli and coated pits is distinct or similar and to what extent it overlaps with other structures such as endosomes are unknown at present.

To obtain information on these questions, in this study we have determined the distribution of several membrane proteins on the organelles involved in endocytosis in proximal tubule cells. We have examined not only kidneys from normal rats, but also those from animals treated with sodium maleate in which protein absorption is substantially inhibited and the morphology of the endocytic compartments is dramatically altered (7).

Materials and Methods

Materials

Male Sprague-Dawley rats (175–200 g) were obtained from Camm Research Lab Animals (Wayne, NJ). Horseradish peroxidase (HRP),[1] Type VI, and paraformaldehyde were purchased from Sigma Chemical Co. (St. Louis, MO). Rhodamine-conjugated sheep anti-mouse IgG was from Cappel Laboratories, Inc. (Cochranville, PA), fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG from Miles Laboratories Inc. (Naperville, IL). Goat anti-rabbit IgG coupled to colloidal gold (5 nm) was from Janssen Life Science Products (Piscataway, NJ). The sources of other reagents were the same as given previously (23, 37).

[1] *Abbreviations used in this paper:* DAB, diaminobenzidine; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase.

Sources of Antibodies

Anti-clathrin antiserum (anticlathrin HC + LC), which detects both clathrin heavy (180 kD) and light (~35 kD) chains (27), was kindly provided by Dr. Daniel Louvard (Institut Pasteur, Paris).

Monoclonal (clone $D_1 55F_2 1$) and rabbit polyclonal IgG raised against purified gp330 (21) were characterized previously (22). The polyclonal IgG was affinity purified by binding to gp330 covalently cross-linked to Sepharose 4B (22). As previously reported (23), the monoclonal antibody specifically recognizes gp330, whereas the affinity purified polyclonal anti-gp330 IgG recognizes both gp330 and maltase (gp 300), which are structurally related but distinctive glycoproteins; by peptide mapping they share many of the same peptides but show some differences (23) and have a different distribution in the brush border.

Rabbit polyclonal anti-γ-glutamyl transpeptidase antiserum, and anti-130 and anti-94 kD IgGs were kindly provided by A. Miettinen (34). Anti-γ-glutamyl transpeptidase was raised against enzyme purified from detergent extracts of rat kidney cortex digested with bromelin (19). By immune overlay on renal brush border fractions, it reacts with both subunits—~54 and ~21 kD—of γ-glutamyl transpeptidase (data not shown). Anti-130 kD and anti-94 kD IgGs were raised against the respective protein bands eluted from preparative SDS gels of rat kidney microvillar fraction and were affinity purified from antisera on columns of Sepharose covalently linked to protein eluted from the respective gel bands (34). By immune overlay on nitrocellulose strips of kidney microvillar fractions, the anti-130 and anti-94 IgGs reacted with only a single band of the appropriate mobility (34).

Mouse monoclonal anti-lgp120 (clone Ly1C6) was a gift from I. Mellman (Yale University School of Medicine). It was raised against purified lysosomal membranes and has been shown to stain lysosomes in cultured NRK and J774 cells by indirect immunofluorescence and to recognize a 120-kD glycoprotein by immunoprecipitation (26).

Anti-β-glucuronidase IgG was a gift from Dr. David Sabatini (New York University School of Medicine). It was raised against β-glucuronidase purified from rat preputial gland (38).

Anti-HRP IgG (DAKO) was purchased from Accurate Chemical & Scientific Corp., Westbury, NY.

Preparation of Tissue for Immunocytochemistry

Kidneys from control, maleate-treated, or HRP-injected rats were fixed by perfusion with periodate-lysine-paraformaldehyde fixative (31) followed by immersion for 4–6 h in the same fixative. Some tissue pieces were cryoprotected in 2.3 M sucrose, frozen in liquid nitrogen, and used for preparation of semithin cryosections on an ultramicrotome (41). Others were cryoprotected in dimethylsulfoxide, frozen in isopentane and stored in liquid N_2 until sectioned on a cryostat (23).

Immunofluorescence

Semithin (0.5 μm) cryosections were prepared from periodate-lysine-paraformaldehyde-fixed kidney on a Reichert OMU-4 ultramicrotome (Reichert Scientific Instruments, Buffalo, NY) equipped with a cryoattachment using the techniques of Tokuyasu et al. (41). They were incubated simultaneously in a mouse monoclonal antibody—either anti-gp330 IgG (0.5 μg/ml) or anti-lgp120 (1:40), and a rabbit polyclonal antibody—either anticlathrin (HC + LC) antiserum (1:50), anti-gp330 IgG (2 μg/ml), anti-γ-glutamyl transpeptidase antiserum (1:200), anti-130 kD (2 μg/ml), anti-94 kD IgG (2 μg/ml), anti-β-glucuronidase IgG (1:50), or anti-HRP IgG (1:1,000). Incubations were carried out in 0.1% gelatin, phosphate-buffered saline (PBS), 50% goat serum, overnight. After washes in 0.2% gelatin in PBS, the sections were incubated (60 min) simultaneously with rhodamine-conjugated sheep anti-mouse IgG (1:50) to detect the monoclonal IgGs and FITC-conjugated goat anti-rabbit IgG (1:60) to detect polyclonal IgGs. They were then washed again and mounted in 50% glycerol in PBS containing phenylenediamine (to reduce fading) (20). Micrographs were taken on a Zeiss Photomicroscope II equipped with epifluorescence optics using Kodak Tri-X Pan film, ASA 400.

Immunogold

Cryostat sections (15–20 μm) of fixed kidney were incubated with polyclonal anti-gp330 IgG (2 μg/ml), rabbit anti-γ-glutamyl transpeptidase antiserum (1:200), anti-130 (2 μg/ml), or anti-94 kD IgG (2 μg/ml) overnight and then incubated overnight in colloidal gold-conjugated goat anti-rabbit IgG (5 nm) and processed for electron microscopy as previously described (23).

Immunoperoxidase

Cryostat sections prepared from control or maleate-treated rats were incubated with monoclonal anti-gp330 IgG (2 μg/ml), anti-γ-glutamyl transpeptidase antiserum (1:200), anti-130 kD IgG (2 μg/ml), anti-94 kD (2 μg/ml) IgG, anti-clathrin IgG (1:50), or rabbit polyclonal anti-gp330 IgG followed by either a sheep anti-mouse (in the case of monoclonal anti-gp330 only) or a sheep anti-rabbit Fab-peroxidase conjugate. The tissue was postfixed in glutaraldehyde, incubated in diaminobenzidine (DAB) medium, and processed for electron microscopy (5, 23).

Loading Endosomes with HRP

Rats were injected via the femoral vein with 10 mg HRP as described by Straus (40), and the kidneys were fixed (31) by perfusion as described above after 5 or 15 min. Semithin cryosections (0.5 μm) were prepared and incubated with anti-HRP IgG overnight followed by FITC conjugated goat anti-rabbit IgG for indirect immunofluorescence. Nonfrozen sections (30–40 μm) were prepared on a Sorvall TC-2 tissue chopper (Sorvall Instruments Div., E.I. DuPont de Nemours & Co., Newtown, CT) and incubated in DAB medium to detect HRP (16) and processed for electron microscopy.

Sodium Maleate Treatment

Rats were injected with sodium maleate (400 mg/kg) via the formal vein (7), and the kidneys were fixed (31) by perfusion after 60 min.

Results

Organization of the Proximal Tubule Cell

The endosomal compartments of the proximal tubule epithelial cell include apical vesicles and so-called dense apical tubules (8, 30, 42) (Fig. 1). After uptake in coated pits, tracers such as ferritin (9) and HRP (16) are detected sequentially in these compartments (Fig. 1 *b*) and ultimately are observed in lysosomes. The apical tubules resemble similar tubules often seen in continuity with vacuolar endosomes in other cell types (13, 18, 28, 42); however, they are more abundant and are distinctive in that they possess a thick intraluminal coat. Also, connections between the dense apical tubules and apical vesicles or vacuoles are relatively infrequent, suggesting that they are not very numerous or are transient in nature (16, 30, 42). It has been proposed, as believed for other cell types (13), that the tubules may serve to deliver absorbed proteins to the apical vesicles and to recycle membrane components taken up by endocytosis back to the plasmalemma (6, 42).

Double Labeling of Semithin Cryosections by Indirect Immunofluorescence

We have previously shown that the distribution of antigens on the microdomains of the proximal tubule brush border, i.e., whether restricted largely to the microvilli or largely to the coated pits, or present on both plasmalemmal microdomains, can be distinguished by indirect immunofluorescence in semithin cryosections (23, 37). Both clathrin and gp330 were found to be concentrated in the coated pits located at the base of the microvilli, whereas maltase was restricted to the microvilli. To compare directly the localization of two antigens in the same section, we developed a double labeling procedure using three previously characterized antibodies to apical plasmalemmal antigens—anti-clathrin (HC + LC) and monoclonal and polyclonal anti-gp330. It was previously shown that the monoclonal antibody stains only the coated pits at the base of the microvilli because it recognizes only gp330, whereas the rabbit polyclonal anti-gp330 stains the microvilli as well as the coated pits because it reacts with both gp330 and maltase (23).

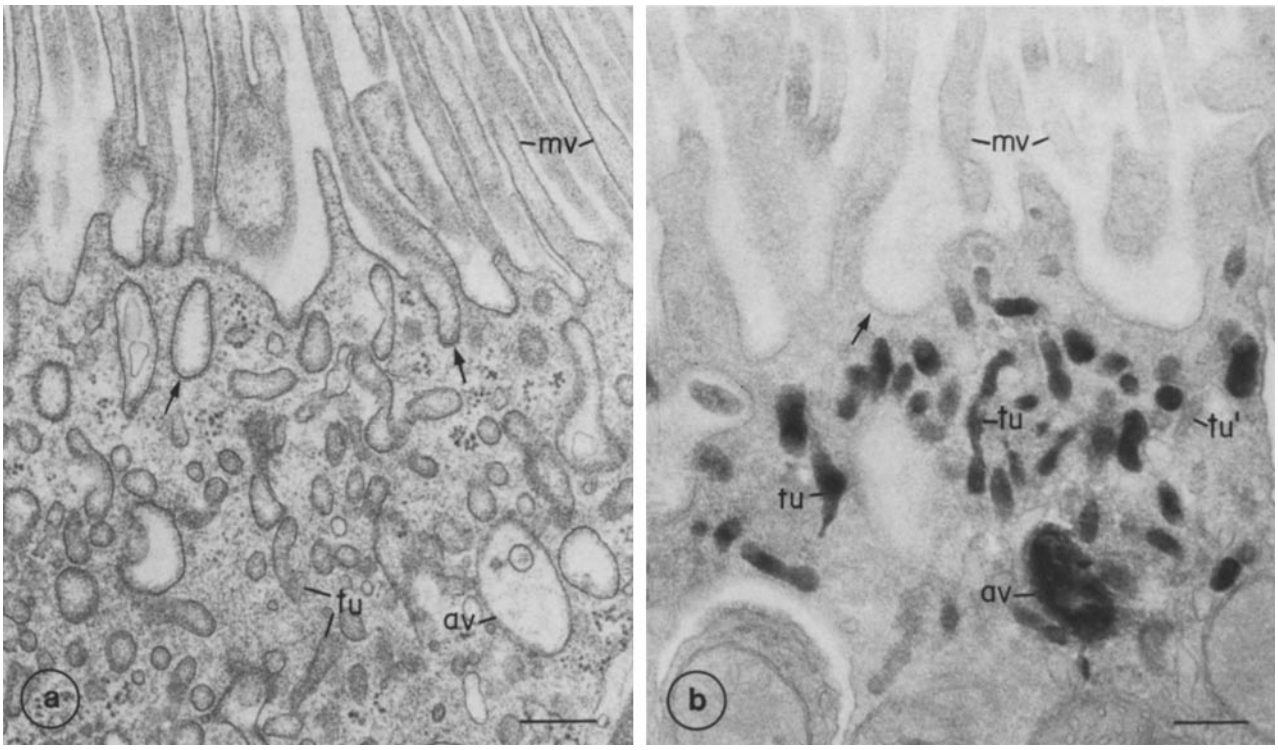

Figure 1. (*a*) Electron micrographs of the apical region of a kidney proximal tubule cell showing the endocytic apparatus, i.e., the coated pits (arrows), apical vesicles (*av*), and dense apical tubules (*tu*). The coated pits have a typical cage like clathrin coat (37). The one on the left is cut so that its continuity with the lumen is not visible in the plane of the section. The apical tubules lack clathrin coats but have a dense internal coat. *mv*, microvilli. (*b*) Similar section of a proximal tubule cell from the kidney of a rat given HRP by intravenous injection 15 min before fixation. The specimen was incubated in DAB medium and processed for electron microscopy. Reaction product is present in apical vesicles (*av*) and in most (*tu*) but not all (*tu'*) of the dense apical tubules. Since the kidney was flushed by perfusion before fixation, reaction product is not detected in the tubule lumen or on membranes of the microvilli (*mv*) or coated pits (arrow). Bars: (*a*) 0.31 μm, (*b*) 0.25 μm.

When a section of rat kidney was doubly incubated with rabbit anti-clathrin (HC + LC) and mouse monoclonal anti-gp330 and was subsequently reacted simultaneously with two different fluorescent conjugates (FITC goat anti-rabbit IgG and rhodamine sheep anti-mouse IgG), the results were the same as obtained with single incubations: the base of the microvilli was stained by both antibodies (Fig. 2, *a* and *a'*). In addition, with anti-clathrin (HC + LC), a punctate staining was detected throughout the cytoplasm (Fig. 2*a*). The punctate cytoplasmic staining is the typical pattern of clathrin staining seen in other cell types (1, 37). It was not detected previously (37), and its detection in this study probably reflects the use of increased concentrations of antibody.

When fixed kidney sections were simultaneously reacted with mouse monoclonal anti-gp330 IgG and rabbit polyclonal anti-gp330 IgG detected with the appropriate fluorescent conjugates, staining with the former was restricted to the base of the microvilli (Fig. 2*b*) and staining with the latter was found in both microvilli and coated pits (Fig. 2*b'*).

We conclude that double labeling by indirect immunofluorescence allowed us to (*a*) determine whether the distribution of specific antigens was largely restricted to the coated pits or restricted to microvilli or was found on both these microdomains; and (*b*) compare the distribution of two different antigens in the same section.

γ-Glutamyl Transpeptidase and the 130- and 94-kD Polypeptides Are Restricted to Microvilli

When anti-γ-glutamyl transpeptidase antiserum (Fig. 2*c'*) or anti-130 or anti-94-kD IgG (not shown) was reacted on kidney sections simultaneously with mouse monoclonal anti-gp330 IgG, by indirect immunofluorescence intense staining was seen along the microvilli with all three polyclonal antibodies. The coated pits were not stained because the signals did not overlap with that of monoclonal anti-gp330 IgG, our coated pit marker (compare Fig. 2, *c* and *c'*). Thus, all three of these polypeptides appeared to be restricted to the microvilli.

Immunoelectron microscopy confirmed that this is the case because after immunogold labeling with anti-γ-glutamyl transpeptidase (Fig. 3*a*), anti-130 kD (Fig. 3*b*), or anti-94-kD (Fig. 3*c*), >95% of the gold particles were localized on microvillar membranes and only 1–3% were found in the coated pits (Table I). Similarly, after immunoperoxidase staining dense deposits of reaction product were observed on the microvilli, but not on the coated pits with these antibodies (data not shown).

By contrast after immunogold labeling with rabbit polyclonal anti-gp330 (Fig. 3*d*), gold particles were detected on both the microvillar (65%) and coated pit (35%) membranes (Table I), and with the immunoperoxidase procedure, heavy deposits of DAB reaction product were concentrated on both the microvilli and coated pits (not shown).

Thus, all the brush border antigens tested are enriched on microvillar membranes and are absent or present in very low concentrations in coated pits.

Identification of Endosomes by Loading with HRP

To identify endosomes, we loaded them with HRP as done

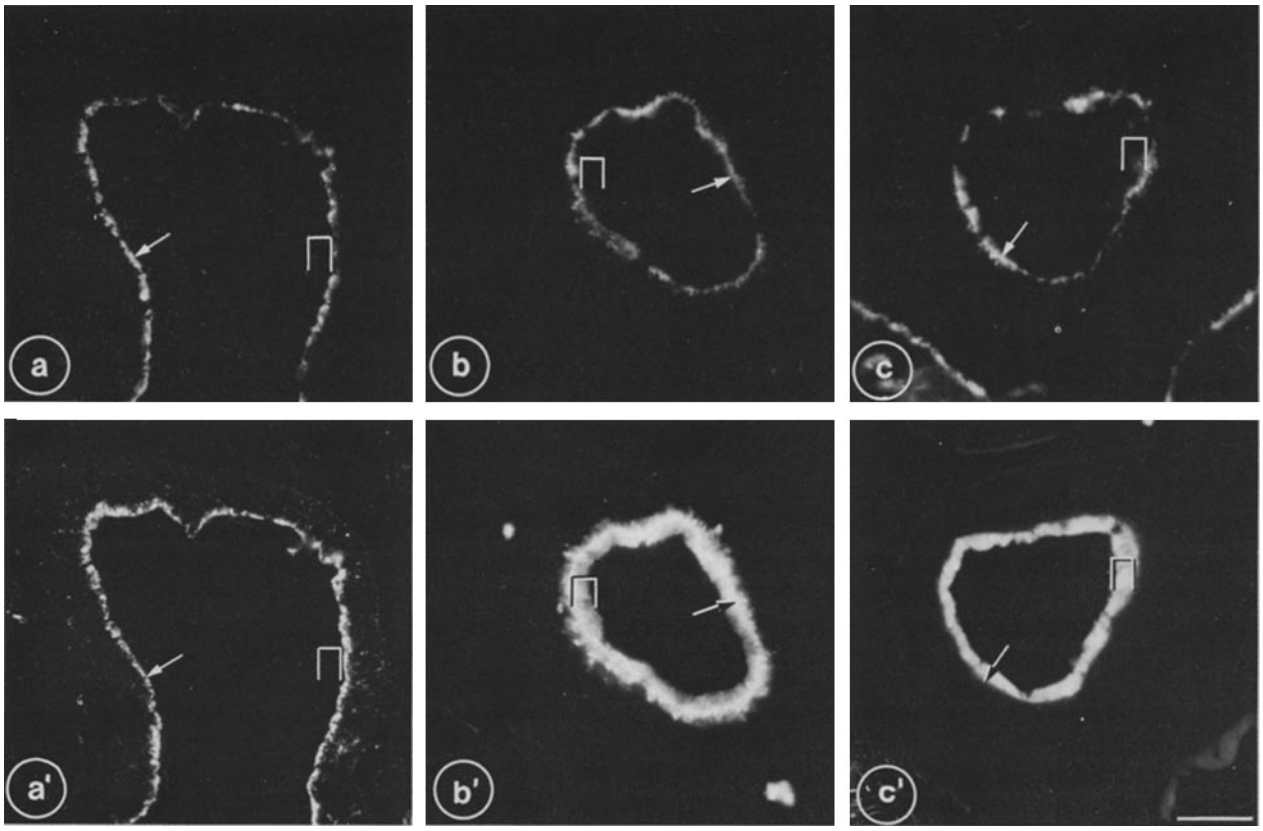

Figure 2. Indirect immunofluorescence of semithin (0.5 μm) cryosections demonstrating simultaneous labeling of rat kidney sections with two antibodies and the resolution that can be obtained in this type of preparation. Sections were incubated simultaneously with mouse monoclonal anti-gp330 (*a–c*), and either rabbit polyclonal anti-clathrin (HC + LC) (*a'*), anti-gp330 IgG (*b'*), or anti-γ-glutamyl transpeptidase (*c'*) followed by rhodamine-conjugated sheep anti-mouse IgG to detect monoclonal anti-gp330 and FITC-conjugated goat anti-rabbit IgG to detect the other antibodies. Identical fields are shown in lettered pairs (e.g., *a* and *a'*). With monoclonal anti-gp330 (*a–c*) and anticlathrin (*a'*), staining is concentrated in coated pits at the base of the microvilli (arrow). With polyclonal anti-gp330 (*b'*), both the microvilli (⊓) and coated pits (arrows) are stained because this antibody recognizes both gp330 and maltase. In contrast, with anti-γ-glutamyl transpeptidase antiserum (*c'*) the microvilli (⊓) are stained, but the bases of the microvilli (arrow) do not appear to be stained (compare *c* and *c'*). Bar, 29 μm.

by Straus, who found that when HRP was injected intravenously, it was taken up by the proximal tubule cells and was found in "apical vesicles" (detected by light microscopy) up to 30 min thereafter (40). These vesicles did not contain acid phosphatase activity, used as a lysosomal marker, and thus correspond to our present definition of endosomes (17).

In kidney sections from rats injected with HRP 15 min before fixation and incubated in DAB medium to detect the tracer, reaction product was seen by light microscopy (Fig. 4) to be distributed in a punctate pattern in the apical cytoplasm of proximal tubule cells somewhat below the level of the microvilli and coated pits. A similar distribution was seen when HRP was detected using an anti-HRP antibody (see Fig. 5*a*). By electron microscopy, HRP was found in small and large apical vesicles and in dense apical tubules (Fig. 1*b*), corroborating that these are endocytic compartments.

Gp330 Is Not Detected in HRP-loaded Endosomes

When specimens obtained from HRP-injected rats were doubly reacted with rabbit anti-HRP IgG and mouse monoclonal anti-gp330 IgG, there was little or no overlap in the pattern of staining (Fig. 5, *a* and *a'*). An intense punctate staining of the apical cytoplasm was obtained with anti-HRP (Fig. 5*a*) that was slightly more basal and distinct, for the most part, from that obtained with mouse monoclonal anti-gp330 IgG (Fig. 5*a'*). Identical results were obtained on specimens fixed at either 5 or 15 min after administration of HRP. Thus gp330, a coated pit protein, is not detectable in HRP-loaded endosomal compartments under these conditions.

Identification of Lysosomes: Co-distribution of β-Glucuronidase and lgp120

When kidney sections were reacted with anti-β-glucuronidase, used as a marker to identify lysosomes, large fluorescent lysosomes were detected throughout the cytoplasm in all but the most apical regions of the cell (Fig. 5*b*). This pattern was similar to that obtained by others when the distribution of lysosomes was determined using histochemical markers for lysosomal enzymes such as acid phosphatase (40). When kidney sections were doubly stained with mouse monoclonal anti-lgp120 and rabbit anti-β-glucuronidase, the fluorescent signal for lgp120 appeared as rings of various sizes which coincided with the lysosomes identified by staining with anti-β-glucuronidase (Fig. 5, *b* and *b'*). Since the same structures were stained with anti-lgp120 and anti-β-glucuronidase, we conclude that, as is the case in cultured cells (26), anti-lgp120 is a valid lysosomal membrane marker in the proximal tubule.

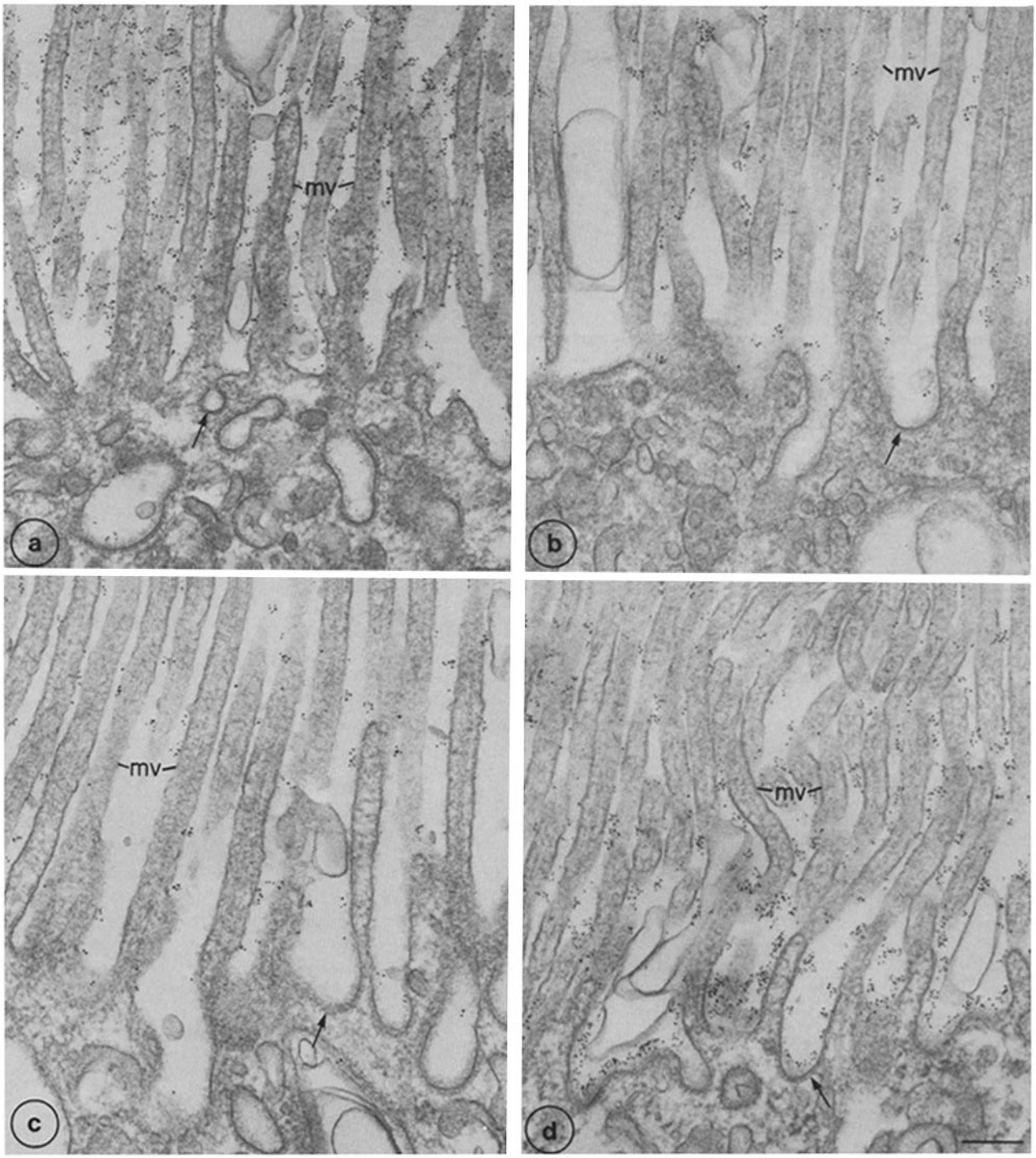

Figure 3. Immunogold labeling of kidney sections with rabbit anti-γ-glutamyl transpeptidase (*a*), anti-130 (*b*), anti-94 (*c*), and anti-gp330 (*d*). With the first three antibodies (*a–c*), gold particles are largely restricted (~95%) to the microvilli (see Table I). With polyclonal anti-gp330 (*d*), gold particles are present on both the microvilli (*mv*) and coated pits (arrows) as previously shown (23). Bar, 0.22 μm.

Lgp120 Is Not Detected in Endosomes or in Coated Pits

The availability of a lysosomal membrane marker allowed us to compare the staining of endosomes with that of lysosomes by immunofluorescence. When kidney sections containing HRP-loaded endosomes were doubly reacted with rabbit anti-HRP IgG and anti-lgp120 (Fig. 5, *c* and *c'*), most of the HRP-containing endosomes concentrated in the apical domain of the cell were not stained with anti-lgp120. Similarly, in preparations doubly labeled with polyclonal rabbit anti-gp330 and anti-lgp120, the staining patterns did not coincide (Fig. 5, *d* and *d'*). Thus by indirect immunofluorescence the endosomes and the coated pits did not contain detectable amounts of lgp120, indicating that this protein is restricted to lysosomal membranes (Table II).

*Table I. Percentage of Gold Particles on Coated and Microvillar Microdomains of the Renal Brush Border**

Antibodies	% Gold particles		
	Coated pits	Microvillar membranes	Particles counted
Polyclonal anti-gp330	35.8	64.2	16,067
Monoclonal anti-gp330‡	86.2	13.8	2,000
Monoclonal anti-maltase‡	5.4	94.5	2,000
Anti-γ-glutamyl transpeptidase	1.7	98.3	11,692
Anti-130-kD polypeptide	3.4	96.5	9,962
Anti-94-kD polypeptide	2.7	97.2	9,383

* Cryostat sections were incubated with a specific antibody followed by protein A or goat anti-rabbit IgG conjugated to colloidal gold. Counts of gold particles were made on electron micrographs enlarged 45,000×.
‡ For reference, quantitation of gold particles with monoclonal anti-gp330 IgG and anti-maltase IgG, reported by Kerjaschki et al. (23), are included.

Redistribution of Markers After Sodium Maleate Treatment

We next determined the distribution of the various markers in the proximal tubules of rats treated with sodium maleate. This treatment is known to drastically reduce protein absorption and to cause several metabolic changes (decreased ATP concentration and [Na^+K^+] ATPase activity [24]) in kidney proximal tubules. These physiologic changes are associated with extensive alterations in the endocytic apparatus of the proximal tubule cell (7): the height of the epithelium is reduced, large vacuoles accumulate in the apical cytoplasm (Fig. 6), and most of the dense apical tubules disappear. The microvilli do not appear to be affected by this treatment (7), but its effect on coated pits has not been examined.

To determine whether or not the distribution of microvillar membrane markers was altered, sections from kidneys of maleate-treated rats were doubly labeled with anti-γ-glutamyl transpeptidase and monoclonal anti-gp330 IgG (Fig. 5, *e* and *e'*). With the former, the microvilli were intensely stained (Fig. 5, *e*), but the broad apical region at the base of the microvilli where gp330 is localized (Fig. 5 *e'*) was not stained. By immunoperoxidase, dense reaction product was detected on the microvilli but not on the newly formed vacuoles with anti-γ-glutamyl transpeptidase (Fig. 7 *c*). Identical results were obtained with anti-130- and anti-94-kD IgG (not shown). The

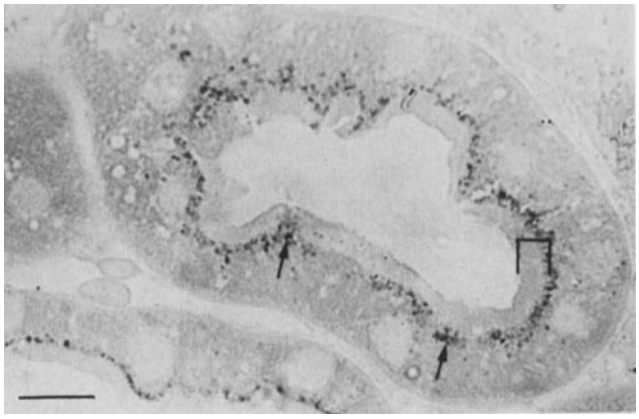

Figure 4. Localization of HRP in the rat kidney proximal tubule 15 min after intravenous injection. Preparation was the same as in Fig. 1 *b*. In this semithin (0.5 μm) section, reaction product (arrows) is seen in the apical region of the cell just beneath the microvilli (⊓) and coated pits. Bar, 25 μm.

fact that the microvillar membrane proteins remained restricted to microvilli supports the previous conclusion, based on morphologic findings (7), that the membrane incorporated into the newly formed cytoplasmic vacuoles is not derived from microvillar membranes.

In sections incubated with monoclonal (Fig. 5 *e'*) or polyclonal (Fig. 5 *f*) anti-gp330 it was apparent even by light microscopy that there had been a change in the distribution of gp330: by both immunofluorescence (Fig. 5, *e'* and *f*) and immunoperoxidase (Fig. 6 *a*) the distribution of gp330 was broader and extended deeper into the apical cytoplasm than in controls (see Fig. 2, *a–c* and *b'*). By electron microscopy, gp330 was detected in many of the newly formed apical vacuoles (Fig. 7 *b*). This pattern is quite different from that observed in normal kidney (Fig. 7 *a*), where gp330 is largely restricted to the coated pits at the base of the microvilli and is not detected in endocytic compartments.[2]

The distribution of clathrin also changed after maleate

[2] We previously reported the presence of gp330 in vacuolar structures thought to be endosomes (23). The present results obtained using endocytic markers demonstrate that this is unusual because co-localization of gp330 with endocytic markers is an infrequent finding.

Figure 5. Double indirect immunofluorescence of the rat proximal kidney tubule simultaneously incubated with a rabbit polyclonal and a mouse monoclonal antibody followed by simultaneous incubation in anti-rabbit fluorescein conjugate and an anti-mouse rhodamine conjugate. Paired letters show identical fields. The location (or would-be location) of the same structure is indicated by matching arrows in both figures. (*a* and *a'*) Identification of HRP-loaded endosomes and comparison of their distribution with that of gp330. Sections from rats injected with HRP (15 min before they were killed) and doubly incubated with anti-HRP (*a*) and monoclonal anti-gp330 (*a'*). Note that in general the punctate pattern of endosomal staining seen with anti-HRP (arrows in *a*) is located slightly deeper in the apical cytoplasm than the coated pits at the base of the microvilli stained with anti-gp330. (*b* and *b'*) Identification of lysosomes in sections doubly incubated with anti-β-glucuronidase (*b*) and anti-lgp120 (*b'*). The same lysosomal structures (arrows) are stained with both antibodies except that with anti-lgp120, staining is limited to the periphery of lysosomes which appear as fluorescent rings. (*c* and *c'*) Comparative distribution of endosomes (arrows) detected by anti-HRP (*c*) and lysosomes demonstrated by anti-lgp120 (*c'*). Note that there is little or no overlap between the two signals: endosomes are located more apically than lysosomes. (*d* and *d'*) Distribution of lysosomes in a section doubly incubated with anti-gp330 (*d*) and anti-lgp120 (*d'*). The staining patterns are distinctive: there is little or no staining of the microvilli or coated pits with anti-lgp120 and no staining of lysosomes for anti-gp330 (arrows). (*e*, *e'*, *f*, and *f'*) Localization of membrane markers in proximal tubules from kidneys of rats treated with sodium maleate as described in Materials and Methods. Sections were incubated simultaneously with anti-γ-glutamyl transpeptidase (*e*) and monoclonal anti-gp330 (*e'*), or polyclonal anti-gp330 (*f*) and anti-lgp120 (*f'*). In contrast to controls (*a'* and *d*), a broad band of apical cytoplasm (arrows) is intensely stained with both monoclonal (*e'*) and polyclonal (*f*) anti-gp330. However, this region is not stained either with anti-γ-glutamyl transpeptidase (*e*), which is confined to the microvilli (⊓) or with anti-lgp120 (*f'*), which is restricted to lysosomes. The microvilli (⊓) are also stained wtih the polyclonal anti-gp330 as in controls (*d*). Bar, 29 μm.

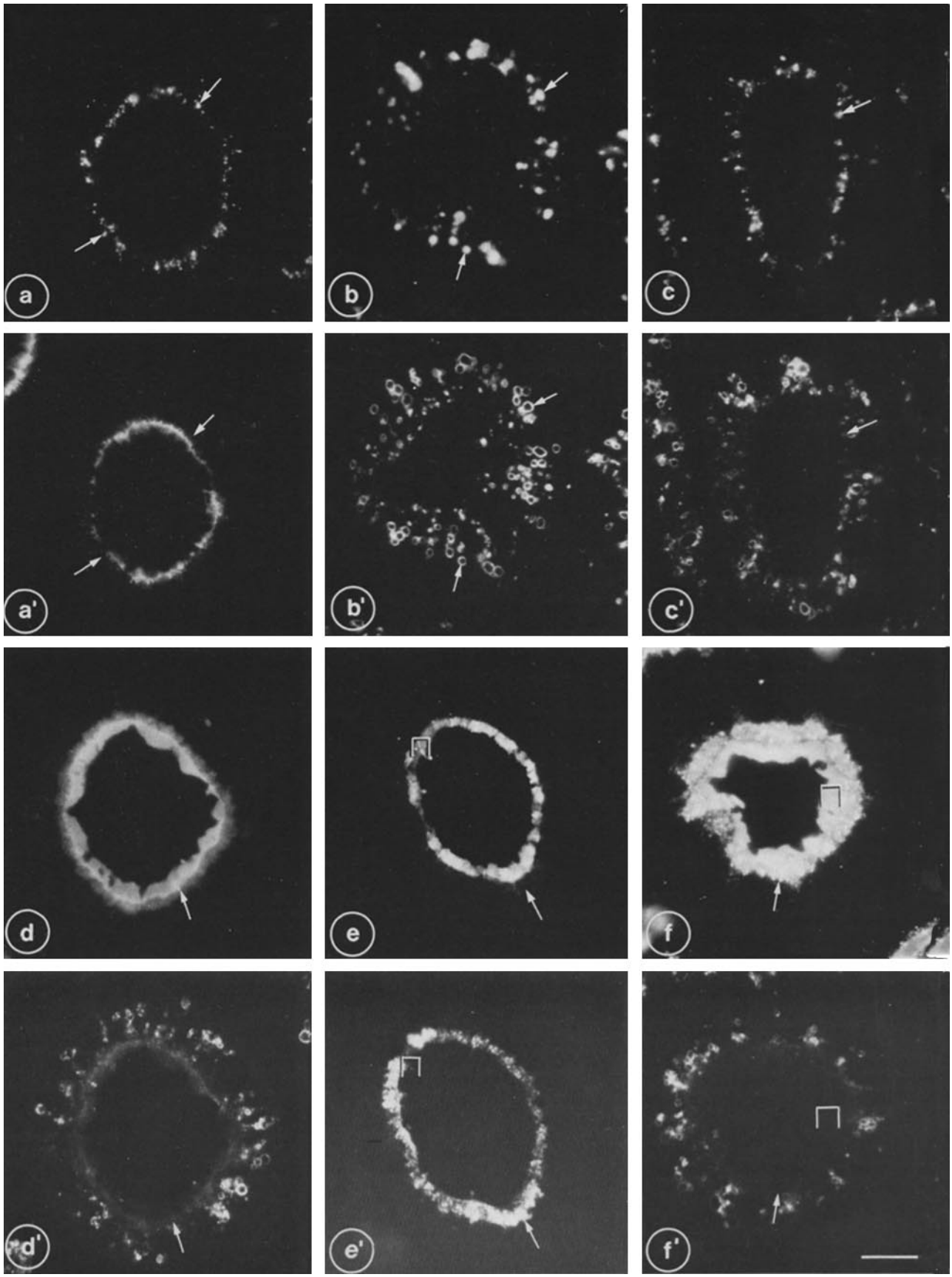
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Table II. Distribution of Marker Proteins in Proximal Tubule Epithelium

Antibodies	Microvilli	Coated pits	Endosomes*	Lysosomes
Anti-gp330 (polyclonal)	+	+	–	–
Anti-gp330 (monoclonal)	–	+	–	–
Anti-γ-glutamyl transpeptidase	+	–	–	–
Anti-130-kD polypeptide	+	–	–	–
Anti-94-kD polypeptide	+	–	–	–
Anti-HRP	–	–	+	–
Anti-lpg120	–	–	–	+
Anti-β-glucuronidase	–	–	–	+

* Includes apical vesicles and dense apical tubules.

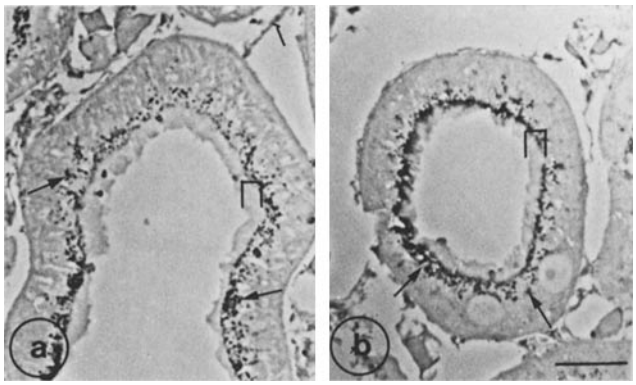

Figure 6. Immunoperoxidase staining with monoclonal anti-gp330 (*a*) and anticlathrin (*b*) in proximal tubules of a maleate-treated rat. The apical cytoplasm appears vacuolated after this treatment. In both figures reaction product is not restricted to the coated pits at the base of the microvilli as in controls (23, 37), but it is broadly distributed in the apical cytoplasm (arrows) in the region where the newly formed vacuoles are located. ⊓, microvilli. Semithin (0.5 μm) section cut from a cryostat section embedded in Epon. Bar, 33 μm.

treatment: by light microscopy (Fig. 6*b*) it was clear that instead of being restricted to the base of the microvilli as in controls, staining appeared in a broad region of the apical cytoplasm. By electron microscopy reaction product was found either diffusely distributed in the apical cytoplasm or associated with small apical coated vesicles or as empty clathrin cages (Fig. 7*d*). Staining of apical coated pits was much less frequent and less intense than in controls. These findings suggest that the apical vacuoles that appear after maleate treatment are formed as a result of the internalization of coated pits marked by gp330, and these membranes lose their clathrin coats upon internalization. The reduced ATP concentration or other metabolic changes induced by maleate treatment (24) may promote disassembly or prevent reassembly of the clathrin coated pits (25, 33).

With mouse anti-lgp120 (Fig. 5*f'*), lysosomes were seen to be located in the middle or basal regions of the cell as in controls. The lysosomal membrane antigen was not detected in the apical region of the proximal tubule, indicating that the membrane from the newly formed vacuoles is not derived from lysosomal membranes.

Discussion

In this study we have determined the distribution of several membrane proteins in the microdomains of the apical plasmalemma and in endosomes and lysosomes of the proximal tubule cell. The findings, summarized in Table II, indicate that there is little or no overlap in the distribution of the markers tested among these structures because (*a*) three major brush border proteins (γ-glutamyl transpeptidase and the 130- and 94-kD polypeptides) were restricted to microvilli and were absent from coated pits; (*b*) the coated pit marker, gp330, was confined to coated pits and was not detected in either endosomes or lysosomes; (*c*) the lysosomal membrane protein, lgp-120 (26), was not found in any structure except lysosomes (identified with anti-β-glucuronidase); and (*d*) endosomes (identified by HRP loading) lacked detectable staining for any of the membrane markers tested. It can be concluded that the membranes of the microvilli, coated pits, endosomes, and lysosomes have a distinctive protein composition with respect to the markers tested.

Previously it was proposed based on the finding that the membranes of the microvilli and coated pits differ with respect to the distribution of gp330 and maltase, that these microvillar and coated pit membranes constitute plasmalemmal microdomains of distinctive composition (23). This conclusion is strengthened by the present findings demonstrating that three more brush border proteins—γ-glutamyl transpeptidase, and the 130- and 94-kD polypeptides—are confined to the microvilli. Since these three proteins represent major protein bands present in SDS-gels of microvillar fractions, it can be concluded that microvillar membrane proteins differ significantly from those of coated pits. So far, the only known marker for the coated pits of the proximal tubule cell is gp330, the Heymann nephritis antigen (21–23). The function of this protein in the coated pits of the glomerular and proximal kidney tubule epithelia and in several other absorptive epithelia (yolk sac, epididymis) where it has been detected (11) is unknown. It is striking that in this study we found that gp330, unlike other coated pit markers such as LDL (2), asialoglycoprotein (13), and transferrin (18, 43) receptors, is not detected in endocytic compartments, suggesting that it remains at the cell surface and is not transferred to endosomes during endocytosis. Only under pathologic conditions such as maleate treatment is this marker internalized by the proximal tubule epithelium. This being the case, gp330 must represent a constitutive component of these coated pits, its function for the moment remaining unknown.

Recently, there has been considerable interest in determining the comparative composition of the membranes of coated pits, endosomes, and lysosomes since these structures are in functional continuity during endocytosis. Several investigators have analyzed the membrane composition of these compartments in different systems and conflicting results have been obtained (3, 4, 10, 32, 36). Based on results obtained after iodination of the cell surface and endosomal membranes in the J774 macrophage cell line, the plasmalemma and endosomal membranes appeared to be almost identical (32). However, the analysis by SDS PAGE of enriched cell fractions obtained from the rat kidney proximal tubule (3, 4), the rat liver (36), and a human fibroblast cell line (10), it was concluded that the composition of endosomal membranes differs from that of the plasmalemma. Our studies on both control and maleate-treated kidneys indicate that the composition of endosomal membranes differs from that of the microvillar, coated pit, and lysosomal membranes because none of the

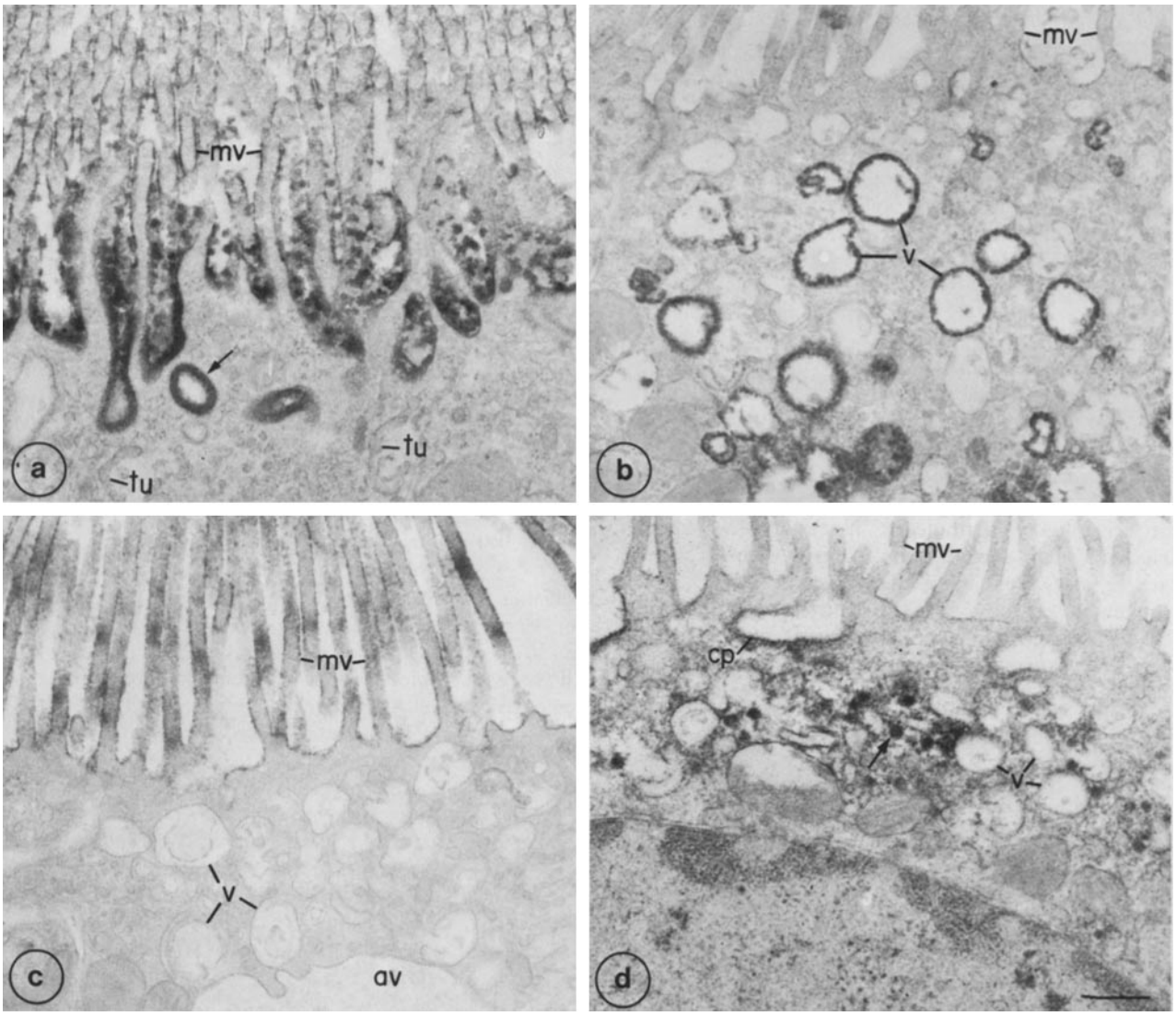

Figure 7. Immunoperoxidase staining of proximal tubule cells from a control kidney reacted with polyclonal anti-gp330 (*a*) or from kidneys of maleate-treated rats reacted with monoclonal anti-gp330 (*b*), anti-γ-glutamyl transpeptidase (*c*), or anti-clathrin (*d*). After maleate treatment, most of the large apical vacuoles (*v*) are seen to contain gp330. This pattern can be contrasted to that of the normal kidney (*a*) where reaction product is restricted to microvilli and coated pits (arrow) with polyclonal anti-gp330 and is not present on apical tubules (*tu*) or in other organelles. With anti-γ-glutamyl transpeptidase (*c*), dense reaction product is present on the microvilli as in controls, but it is not detected in the newly formed vacuoles (*v*). With anti-clathrin (*d*), reaction product is diffusely distributed in the apical cytoplasm or is concentrated on cages of small coated vesicles (arrow) after maleate treatment. Occasionally reaction product is detected on a coated pit (*cp*), but, when present, it is less intense than in the normal kidney. Bar, 0.47 μm.

antigens tested, including the coated pit marker gp330, co-localized with endosomes marked with HRP. Since the function of endosomes is unique and distinct from that of the coated pits and lysosomes (17), it seems probable that some presently undefined proteins are restricted to endosomal membranes. Differences probably also exist between the membrane composition of structurally distinct endosomal compartments, i.e., the apical vesicles and dense apical tubules. Our results demonstrating that the antigens tested are differentially distributed and are restricted to, or concentrated on specific membranes, clearly supports the observations of Bode et al. (3, 4), which suggests that the polypeptide composition of the plasmalemmal, endosomal, and lysosomal membranes of the rat kidney proximal tubule are distinctive.

A further finding was that after maleate-treatment, gp330, which normally is confined to clathrin-coated pits at the cell surface, is found inside the cell in newly formed apical vacuoles, and clathrin is not associated with these membranes but is found either on small coated vesicles, as empty cages, or diffusely distributed in the apical cytoplasm. These findings suggest that the clathrin coats disassemble and plasmalemmal coated pits are internalized after maleate treatment. It has recently been shown (15) that clathrin can exist in both a membrane-associated and a "soluble" form and that the relative amounts of the two forms varies among different cell types. Cage assembly is known to be affected by the metabolic state of the cells and, more precisely, disassembly is promoted by decreased K^+ (25) and ATP (33) levels in intact cells. In cell-free systems, ATP and an uncoating ATPase have been shown to stimulate cage dissociation (39), however, the precise

function of ATP in the clathrin assembly/disassembly cycle in vivo is not clear (39). The simplest explanation of our findings is that maleate treatment, which causes a reduction in ATP levels in the proximal tubule (24), promotes disassembly or prevents assembly of clathrin coats on the coated pits.

The fact that gp330 is not normally detected in the endosomal compartments but is restricted to the coated pits has bearing on the question, Are the components of coated membranes in the proximal tubule cell selectively internalized during endocytosis? Most investigators favor the idea that coated pits pinch off the plasma membrane to form coated vesicles which serve to ferry surface bound ligands and fluid phase components to endosomes (14, 17). However, Pastan and Willingham (35) have proposed that coated pits remain stationary at the cell surface and that ligands and fluid-phase components are internalized and delivered to endosomes (which they call "receptosomes" [44]) via uncoated tubules. Our results indicating that gp330 normally remains at the surface of the proximal tubule cell and is not present or present in very low concentrations in endocytic compartments suggest that in the untreated kidney this coated pit marker may not be internalized. Alternatively, it may be internalized and rapidly recycled to the plasma membrane. In the latter case, the concentration of gp330 in any of the endosomal compartments could conceivably be too low to be detectable. Surface labeling of gp330 and analysis of its transport during endocytosis is required to distinguish between these two possibilities.

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