Presence of an Extensive Clathrin Coat on the Apical Plasmalemma of the Rat Kidney Proximal Tubule Cell

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ABSTRACT The nature of the cytoplasmic coat present on the apical invaginations of the kidney proximal tubule cell was investigated by immuneoverlay and immunocytochemistry of renal brush borders with anticlathrin antibodies. When kidney cortex was prepared for electron microscopy using methods that enhance visualization of clathrin coats, the apical invaginations at the base of the brush border microvilli were seen to be backed by a nearly continuous coating which resembles but is more extensive than the lattice-like clathrin coats found around brain coated vesicles. When isolated brush border fractions were prepared under conditions that preserve the coats, separated by SDS PAGE, and transferred to nitrocellulose, the presence of clathrin heavy and light chains was detected by immuneoverlay using two different affinity-purified anticlathrin IgGs—one that we prepared, which detects only the clathrin light chains, and the other, prepared by Louvard et al. (Louvard, D., C. Morris, G. Warren, K. Stanley, F. Winkler, and H. Reggio, 1983, EMBO [Eur. Mol. Biol. Organ.] J., 2:1655–1664), which detects both the heavy and light chains. As viewed by light microscopy (immunofluorescence or immunoperoxidase), staining with both anticlathrins was concentrated at the base of the proximal tubule microvilli. Immunoelectron microscopic localizations carried out on brush border fractions (using peroxidase and gold conjugates) demonstrated specific binding of anticlathrin IgGs to the lattice-like cytoplasmic coat. When brush border fractions were reacted with monoclonal antibodies prepared against gp330 and maltase, proteins that serve as markers for the membrane of the apical invaginations and microvilli, respectively (Kerjaschki, D., L. Noronha-Blob, B. Sacktor, and M. G. Farquhar, 1984, J. Cell Biol., 98:1505–1513), the two proteins retained their restrictive distribution in the brush border. The findings demonstrate (a) that the cytoplasmic coat of the proximal tubule intermicrovillar apical invaginations is composed of clathrin heavy and light chains, and (b) that the differential distribution of proteins in these two brush border microdomains is maintained in appropriately prepared brush border fractions.

In the kidney proximal tubule, proteins filtered by the glomerulus are reabsorbed by endocytosis in so-called apical invaginations located at the base of the microvilli along the luminal cell surface (1). Recently it was demonstrated (2) that the membrane of the apical invaginations has a different composition from the membrane of the microvilli: A large membrane glycoprotein, gp330 (the Heymann nephritis antigen [3]) was found to be concentrated in the apical invaginations, whereas gp300 (renal maltase [4]), an immunologically related membrane glycoprotein, is concentrated in the microvilli. The mechanisms by which gp330 is selectively included in the pits and gp300 is selectively excluded are not understood but could involve interactions with cytoskeletal elements. An amorphous coat has been observed on the cytoplasmic surface of the apical invaginations (1) and, although the precise nature of the coat has not been determined, there is suggestive evidence (5) that it might be composed of clathrin.

In this study, the results of which were briefly reported elsewhere (6), we have investigated the molecular nature of the cytoplasmic coat on the apical membrane of the rat kidney proximal tubule cell, and we have obtained immunocytochemical and immunoelectron microscopic evidence that it contains clathrin heavy and light chains. Furthermore, we have found that when brush border fractions are prepared under conditions in which the coats remain assembled, the differential distri-

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bution of gp330 and maltase in the two plasmalemmal microdomains is maintained.

MATERIALS AND METHODS

Materials: Female rabbits (2 kg) were obtained from Charles River Laboratories (Wilmington, MA), and male Sprague-Dawley rats (175-200 g) from Cam Research Laboratory Animals (Wayne, NJ). 2-(N-morpholino)ethane sulfonic acid (MES) (Sigma Chemical Co. (St. Louis, MO). Low melting point agarose was obtained from FMC, Main Colloid Division (Rockland, ME) and [{}]

Preparation of Crude Brain Coated Vesicles and Soluble Coat Protein: A crude coated vesicle fraction was prepared from porcine or rat brain to the first velocity gradient step of Pearse (2). An enriched fraction of soluble coat protein was prepared by dialysis of the crude coated vesicle fraction against 10 mM HEPES buffer, pH 8.5, containing NaN3 (overnight) and centrifugation at 100,000 g (60 min). Purified triskelions were prepared as described by Ungewickell and Branton (8).

Antibodies: Two different polyclonal anticalathrin antibodies were used in this study: one was prepared by injecting soluble coat protein (1 mg) intradermally into rabbits and boosting biweekly. The immune serum was affinity purified on columns of Sepharose covalently linked to purified triskelions. This anti-coat protein IgG detects only the clathrin light chains (see Results and Fig. 2), and thus will be referred to as anti-clathrin (LC). The other anti-clathrin, kindly provided by Dr. Daniel Louvard (Pasteur Institute, Paris), detects both the heavy (180 kd) and light (-35 kd) chains (9), and will be referred to as anti-clathrin (HC + LC).

Other antibodies utilized were a monoclonal anti-gp330 (clone D,55F21) prepared previously (10), monoclonal anti-maltase (clone 1F12GI), a gift from Dr. B. Sacktor (National Institute of Aging, Baltimore) (4), and a polyclonal antibody raised in rabbits against purified gp330 (3). The polyclonal IgG was affinity purified either by binding to gp330 on a column (10) or on a nitrocellulose transfer (11). As previously reported (2), the monoclonal antibodies specifically recognize only gp330 or maltase and were used for immunocytochemical studies, whereas the affinity-purified polyclonal anti-gp330 IgG recognizes both gp330 and maltase (structurally similar glycoproteins) (2) and was used for immunoelectrophoresis.

Immunogold Staining of Isolated Coated Vesicles: A crude fraction of coated vesicles from rat brain was fixed in 1% glutaraldehyde in 0.1 M MES buffer, pH 6.5, 1 mM EGTA, 0.5 mM MgCl2 (2 min) and adsorbed to electron microscope grids. The grids were floated on drops of 50 mM glycine in MES buffer (10 min) to quench free aldehyde groups, washed, and incubated with affinity-purified anti-coat protein IgG (60 min). After washing, they were incubated with Protein A-gold (10 min), washed again, negatively stained with 1% uranyl acetate, and examined in a Siemens 101 electron microscope.

Preparation of Brush Border Fractions: Isolated brush border fractions were prepared according to Thuneberg and Rostgaard (12) except that EGTA (100 mM), and protease inhibitors (1 ug/ml antipain, 100 ug/ml benzamidine, 1 ug/ml pepstatin, 0.2 mM phenylmethylsulfonyl fluoride and diisopropylphosphofluoridate) were added to the homogenization media, and the final washes consisted of 0.5 M sucrose, 0.2 mM EGTA, 0.2 mM MgCl2 in 0.02 M MES, pH 6.5.

SDS PAGE and Immunoelectrophoresis: Crude coated vesicle fractions (rat brain) or kidney brush border fractions were subjected to SDS PAGE on 5-10% acrylamide gels (20 cm long) or 4-10% acrylamide mini gels (4 cm long) in the Maizel buffer system (13) and the proteins were electrophoretically transferred to nitrocellulose (4 h) in the Maizel buffer system (13) and the proteins were electrophoretically transferred to nitrocellulose (4 h) in the presence of 0.1 M Tris and 0.06 M boric acid, pH 8.3 (14). A strip of the gel was reserved for staining with Coomassie Blue; strips of the nitrocellulose transfers were incubated for 4 h in 2% hemoglobin in PBS containing NaN3 (quench buffer) and reacted with affinity-purified anticalathrin or anti-gp330 IgG in quench buffer overnight. After washes in PBS, the paper strips were incubated with [{}]

Preparation of Tissues for Electron Microscopy: For morphologic studies, rat kidneys were fixed by perfusion (2) with 4% formaldehyde and 0.1% glutaraldehyde in MES buffer (0.1 M MES, 1 mM EGTA, 1 mM MgCl2) for 5-7 min, postfixed in unbuffered 2% OsO4 with 1 mM MgCl2 on ice for 48 h, and then stained en bloc for 24 h in 0.5% uranyl acetate in H2O at 4°C (to enhance visualization of the cytoplasmic coat [15]), and processed for electron microscopy. For immunofluorescence, tissues were similarly fixed by perfusion with 4% formaldehyde and 0.1% glutaraldehyde in PBS, and for immunoperoxidase, in periodate-lysine-paraformaldehyde (16).

Preparation of Fractions for Immunocytochemistry: Brush border fractions were fixed in suspension with 4% formaldehyde and 0.1% glutaraldehyde (10 min) or in periodate-lysine-paraformaldehyde (16) (4-6 h) and centrifuged (2000, g for 8 min) at 4°C. The pellet was resuspended (1:1) in the supernatant, mixed (1:1) with 3% agarose in PBS, and cast into a thin layer between glass slides (17). Small pieces were prepared, treated with NH4Cl in PBS for 30 min at 20°C (to quench free aldehyde groups), and used for immunocytochemical incubations.

Immunocytochemistry: For immunofluorescence, frozen sections (0.5 u.m) of fixed rat kidney prepared on a cryo-ultramicrotome (2), were incubated with anti-clathrin (LC) antibody (3 ug/ml) followed by rhodamine-conjugated goat anti-rabbit IgG (diluted 1:50). Micrographs were taken on Kodak Tri-X Pan film, ASA 400, using a Zeiss Photomicroscope II, equipped with epifluorescence optics.

For immunoperoxidase, cryostat sections of aldehyde-fixed kidney cortex or strips of agarose-embedded fractions were incubated sequentially in (a) rabbit anti-clathrin (HC + LC) (1:100) or mouse monoclonal anti-gp330 (0.5 ug/ml) or antimaltase (1:100) antibodies, (b) sheep anti-rabbit or sheep anti-mouse P(ab')-peroxidase conjugates, respectively, and (c) diaminobenzidine medium as detailed elsewhere (2, 18).

For immunogold, strips of agarose embedded fractions were incubated with (a) anti-clathrin (LC) IgG followed by Protein A-gold, or (b) monoclonal anti-gp330 or antimaltase IgG, rabbit anti-mouse IgG, followed by Protein A-gold. They were then postfixed in glutaraldehyde and processed as described previously (2).

RESULTS

Characterization of Anticalathrin (LC) IgG

When the affinity-purified, anti-coat protein IgG we prepared was used to indirectly stain isolated coated vesicles with

**FIGURE 1** Indirect immunogold staining of isolated brain coated vesicles. A crude coated vesicle fraction was adsorbed onto an electron microscope grid and incubated with our affinity-purified (by binding to triskelions) anti-coat protein IgG followed by Protein A-gold. They were then postfixed in glutaraldehyde and processed as described previously (2).

Abbreviations used in this paper: HC, clathrin heavy chain; kd, kilodatons; LC, clathrin light chain; MES, 2-(N-morpholino)ethane sulfonic acid.
Protein A-gold, 90% of the gold particles were localized on coated vesicles (Fig. 1). With a similar amount of nonimmune IgG, much less (~12%) gold was bound, and of that bound, only 18% was localized on coated vesicles (data not shown). Thus the affinity-purified antisera reacted specifically with coated vesicles.

Since the coats of coated vesicles are known to be composed of several distinct proteins, it was necessary to determine the specificity of our antiserum. By immunoblot analysis, affinity-purified IgG reacted with the two ~35-kd bands corresponding to clathrin light chains in a rat brain crude coated vesicle fraction and did not react with the 180-kd clathrin heavy chains (Fig. 2, lanes 1 and 2). Thus, we conclude that the antibody reacts monospecifically with clathrin light chains.

**Presence of an Extensive Cage-like Coating on the Apical Plasmalemma of the Proximal Tubule**

When rat kidney cortex was prepared for electron microscopy using procedures that enhance visualization of coats on the cytoplasmic surface of membranes, a dense coat was observed on the invaginations (Fig. 3) located on the apical plasmalemma at the base of the microvilli. This coat appeared as a lattice-like structure similar to that of clathrin coats observed around isolated brain coated vesicles.

**Immunocytochemical Localization of Clathrin in the Proximal Tubule**

By indirect immunofluorescence, our anticlathrin (LC) IgG intensely stained the apical membrane at the base of the microvilli, but not the microvilli themselves (Fig. 4, A and B). A similar pattern was observed by indirect immunoperoxidase staining with the anticlathrin (HC + LC) IgG prepared by Louvard et al. (9) (Fig. 4 C). These results suggest that the cytoplasmic coat is composed of clathrin.

**Detection of Clathrin Heavy and Light Chains in Brush Border Fractions by Immuneoverlay**

When brush border fractions prepared under conditions known to stabilize the coat on brain coated vesicles (MES...
FIGURE 4  Immunocytochemical localization of clathrin in the kidney proximal tubule. (A and B) Thin frozen sections (0.5 μm) of rat kidney were reacted with anticlathrin (LC) followed by rhodamine-conjugated goat anti-rabbit IgG, and examined by fluorescence (A) or phase-contrast (B) microscopy. (C) 0.05-μm plastic section cut from a cryostat section of rat kidney which had been incubated with anticlathrin (HC + LC) followed by horseradish peroxidase-conjugated sheep anti-rabbit Fab and processed for electron microscopy. Note that in both preparations, staining is concentrated at the base of the microvilli (arrows) where the apical invaginations are located and is not present on the microvilli themselves (brackets). x 320.

buffer, pH 6.5) were analyzed by SDS PAGE, a 180-kd band that co-migrates with rat brain clathrin heavy chain was present (Fig. 2, lane 5). By immuneoverlay analysis, the 180-kd band reacted with anticlathrin (HC + LC) IgG (Fig. 2, lane 6). In addition, two ~35-kd bands were detected with anticlathrin (HC + LC) (Fig. 2, lane 6) and anticlathrin (LC) (Fig. 2, lanes 4 and 7). The 35-kd bands appear as a doublet in 5–10% acrylamide gels (Fig. 2A) and as a single smeared band in the 4–10% acrylamide mini gels used to visualize gp330 and maltase (Fig. 2B). We conclude that both clathrin heavy and light chains are present in our renal brush border fractions.

Detection of gp330 and Maltase in Brush Border Fractions by Immuneoverlay

In immuneoverlays with affinity-purified, polyclonal IgG, both gp330 and maltase were detected in brush border fractions (Fig. 2, lane 8). We used polyclonal antibodies to detect the proteins because of technical problems encountered in utilizing monoclonal antibodies for immunoblotting. The fact that polyclonal IgG recognizes both gp330 and maltase is in accord with previous data and with results obtained by peptide mapping (2), indicating that gp330 and maltase are related proteins that share many antigenic determinants.

Immunocytochemical Localization of Clathrin in Brush Border Fractions

When isolated brush borders were examined by electron microscopy, a lattice-like coat was seen along the cytoplasmic surface of virtually the entire apical cell membrane exclusive of the microvilli (Figs. 5 and 6). In fractions incubated by the indirect immunoperoxidase procedure with either anticlathrin (LC) or anticlathrin (HC + LC) and examined by light microscopy, staining was concentrated at the base of the microvilli (Fig. 7A). By electron microscopy, it was evident that staining was due to heavy deposition of reaction product on the cytoplasmic coats associated with the apical plasmalemma (Fig. 8) at the base of the microvilli. In brush border fractions incubated with anticlathrin (LC) followed by protein A-gold, most (~85%) of the gold particles bound to the coat (Fig. 10).
Immunocytochemical Localization of gp330 and Maltase in Brush Border Fractions

In brush borders incubated with monoclonal anti-gp330, immunoperoxidase staining was seen at the base of the microvilli by light microscopy (Fig. 7B), whereas with monoclonal antimaltase IgG, staining was present on the microvilli (Fig. 7C). By electron microscopy, gp330 was found to be concentrated on the external surface of the coated intermicrovillar membranes by both the immunoperoxidase (Fig. 9) and immunogold (Fig. 11) procedures, whereas maltase was localized to the external surface of microvillar membranes (Fig. 12). The distribution of the two antigens could be quantitated in immunogold preparations where it was found that with anti-gp330, most (65%) of the gold particles were localized on the intermicrovillar membranes (Fig. 11), whereas with antimaltase the majority (94%) bound to the microvilli (Fig. 12).

From these results, we conclude that (a) the apical cytoplasmic coat is composed of clathrin, and (b) the segregation of gp330 and maltase into the intermicrovillar and microvillar microdomains of the brush border is maintained during the fractionation procedures.

DISCUSSION

Our findings demonstrate that the apical plasmalemma of the kidney proximal tubule cell is backed by an elaborate and extensive clathrin coat. That the coat is composed of clathrin is based on the immunocytochemical staining of the coats in kidney tissue in situ and in isolated kidney brush border fractions with two affinity-purified antibodies—one, prepared by us, which reacts monospecifically with the light chains and the other, prepared by Louvard et al. (9), which reacts with both the heavy and light chains.

We assume that the cytoplasmic coat on the apical intermicrovillar membrane is constructed of both clathrin heavy and light chains. The presence of light chains in the coats has been directly demonstrated by immunocytochemistry, using our affinity-purified IgG which in immuneoverlays recognizes only the light chains. The presence of heavy chains can be inferred by the fact that the coat has a polyhedral lattice-like structure similar to that of coated vesicles in other locations, and this coat organization depends on the presence of clathrin heavy chains (19). The presence of clathrin heavy and light chains in kidney brush border fractions has been confirmed in immuneoverlays. In other cell types, e.g., fibroblasts (20) and neuronal tissue (21), clathrin heavy and light chains have been co-localized by immunocytochemistry. They appear to be tightly associated because rather harsh denaturing conditions are required to separate these proteins from one another (8).

The clathrin coat on the proximal tubule apical plasmalemma is much more highly developed and elaborate than those seen in most other locations, e.g., in cultured cells. Comparably elaborate coats have been observed in only a few other cell types, notably in the oocyte (22) where coated vesicles were orginally described (23), and more recently, in the phagocytosing macrophage (24). Both cell types, like the proximal tubule, have highly amplified levels of endocytosis. Although the coat of the proximal tubule cell appears to back all the apical membrane invaginations, it is not clear from two-dimensional images whether or not it consists of a series of closely packed but separate coated pits or a quasi-continuous layer of coated membrane. Three-dimensional imaging...
FIGURES 10-12 Immunogold staining of isolated brush border fractions for clathrin (Fig. 10), anti-gp330 (Fig. 11), and maltase (Fig. 12). In Fig. 10, incubated with affinity-purified anti-clathrin (LC) IgG, most (85%) of the protein A-gold particles (55 out of 64) are localized over the coats (arrow) on the cytoplasmic surface of the apical intermicrovillar membrane. In Fig. 11, incubated with monoclonal anti-gp330 IgG, most (65%) of the particles (53 out of 85) are bound to the outer surface of the apical membrane at the base of the microvilli (arrows). This distribution can be contrasted to that in Fig. 12, incubated with monoclonal antimaltase IgG, where most (94%) of the particles (45 out of 47 of the particles present) are associated with the microvilli. × 45,000.

The renal tubular and intestinal epithelial brush borders have many features in common with regard to the organization and protein composition of their microvilli (25-27). However, cage-like coats are infrequently observed on the intestinal brush border membranes, and clathrin is not visible in SDS gels (25) of intestinal brush border fractions. This undoubtedly reflects the fact that by comparison to the kidney proximal tubule, endocytosis along the luminal surface of the mature rat intestine is quite limited.

A further finding is that brush border fractions can be isolated under conditions (MES buffer, pH 6.5) that maintain assembly of the clathrin coats, and in such preparations, the restricted distribution of membrane constituents in the microvillar and intermicrovillar domains of the apical plasma membrane is largely maintained. As in the case in situ (2), gp330 (the Heymann nephritis antigen [3]) remains concentrated on the coated apical invaginations, and gp300 (maltase) is restricted to microvillar membranes. When we compared counts of gold particles obtained in fractions with those obtained in situ (2) in immunogold preparations, we found that the restrictive distribution of maltase in isolated brush borders is quite comparable to that previously reported in situ (94 vs. 95%), whereas that of gp330 (65 vs. 84%) is less pronounced, but nevertheless significant since the apical invaginations constitute only ~2-5% of the apical cell surface.

Although neither the functional significance nor mechanism of the differential localization of these two proteins is presently understood, it is reasonable to suspect that cytoskeletal elements, including clathrin, may be involved since some proteins are known to be selectively included (28) and other selectively excluded (29) from coated pits. It has been postulated that integral membrane proteins are selectively concentrated in coated pits either by specific interactions with coat proteins (28), or by becoming trapped in the dense meshwork of coat proteins after cross-linkage (e.g., by ligand-receptor interaction) (30).

Renal brush border preparations in which coat assembly can be maintained during the isolation procedure and in which at least two integral membrane glycoproteins (gp330 and renal maltase) remain differentially distributed between the intermicrovillar and microvillar microdomains appear to provide a favorable system for investigating the role of clathrin in maintaining the restrictive distribution of integral membrane proteins.

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