Expression of myosin VI within the early endocytic pathway in adult and developing proximal tubules

DANIEL BIEMESDERFER,1 SUE ANN MENTONE,2 MARK MOOSEKER,3 AND TAMÁ HASSON4

Departments of 1Internal Medicine, 2Cellular and Molecular Physiology, 3Cell Biology, and Pathology, Department of Molecular, Cellular and Developmental Biology, School of Medicine, Yale University, New Haven, Connecticut 06520-8029; and 4Section of Cell and Developmental Biology, Division of Biology, University of California at San Diego, La Jolla, California 92093-0368

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Biemesderfer, Daniel, Sue Ann Mentone, Mark Mooseker, and Tama Hasson. Expression of myosin VI within the early endocytic pathway in adult and developing proximal tubules. Am J Physiol Renal Physiol 282: F785–F794, 2002. First published November 13, 2001; 10.1152/ajprenal.00287.2001.—Myosin VI is a reverse-direction molecular motor implicated in membrane transport events. Because myosin VI is most highly expressed in the kidney, we investigated its renal localization by using high-resolution immunocytological and biochemical methods. Indirect immunofluorescence microscopy revealed myosin VI at the base of the brush border in proximal tubule cells. Horseradish peroxidase uptake studies, which labeled endosomes, and double staining for clathrin adapter protein-2 showed that myosin VI was closely associated with the inter-microvillar (IMV) coated-pit region of the brush border. Localization of myosin VI to the IMV region was confirmed at the electron microscopic level by colloidal gold labeling of ultrathin cryosections. In addition, antigen retrieval demonstrated a small but significant pool of myosin VI on the microvilli. To confirm the association of myosin VI with the IMV compartment, these membranes were separated from other membrane compartments by using 15–25% OptiPrep density gradients. Immunoblotting of the gradient fractions confirmed that myosin VI was enriched with markers for the IMV microdomain of the brush border, suggesting that myosin VI associates with proteins in this compartment. Finally, we examined the expression of myosin VI during nephron development. We found myosin VI present in a diffuse cytoplasmic pattern at stage II (S-shaped body phase) and that it was only redistributed fully to the brush border in the stage IV nephron. These studies support a model for myosin VI function in the endocytic process of the proximal tubule.

endocytosis; clathrin; tubular reabsorption

IN THE KIDNEY, PROTEINS FILTERED by the glomerulus are continuously reabsorbed by receptor-mediated endocytosis in the proximal tubule. The brush border of the proximal tubule is enriched in endocytic receptors, such as megalin, that are involved in the uptake of components from the ultrafiltrate. Although these receptors are present along the length of the microvillus, those that have been characterized are enriched in apical invaginations found between the microvilli (reviewed in Ref. 14). Studies after ligand uptake have confirmed that a ligand is first enriched in these invaginations before traversing through an endosomal compartment and reaching the lysosome (7, 15).

The apical invaginations between the microvilli are enriched in the vesicle coat protein clathrin (4, 40). Calveolin, a coat protein common to endocytic vesicles in many cell types, is not abundant in the apical domain of proximal tubule cells (8), suggesting that clathrin-mediated endocytosis is the predominant form of membrane uptake from the apical domain.

Immediately subjacent to the clathrin-coated-pit region of the proximal tubule cell is a region rich in vesicles and tubules (reviewed in Ref. 14). Electron microscopic studies have confirmed that many of the tubules are continuations of the clathrin-coated pits (6). There are also abundant vesicles, identified as coated and noncoated early and late endosomes, as well as dense apical tubules connected to the endosomes that play roles in receptor recycling (13, 14). Because of the unique topology of the proximal tubule cell, the ligand-bound receptors must traverse down the length of the microvillus to reach the intermicrovillar (IMV) domains before being endocytosed. This model is supported by studies in Madin-Darby canine kidney cells after uptake of cationic ferritin, in which pulse-chase experiments revealed an association of ferritin, first, with microvilli and, later, with apical tubules (25). Given the extensive actin cytoskeleton present in the brush border, it is likely that actin is required for endocytosis in the proximal tubule and that actin-based motors, or myosins, are involved in transporting the ligand-bound receptors down the microvilli into the clathrin-coated IMV zone.

In support of this model, apical uptake by polarized epithelial cells is sensitive to actin-depolymerizing drugs...
However, this role for actin in endocytosis is not universal, because drugs that depolymerize actin have little effect on endocytosis assayed in vivo and in vitro in fibroblasts or nonpolarized cells. Therefore, actin may only be required for endocytosis in polarized cell types with dense apical microvilli.

Several classes of myosin motors have been localized to the brush-border microvilli of proximal tubule cells, and these motors are likely barbed-end directed (12, 17, 44, 46). Microvilli are composed primarily of bundled actin filaments that are polarized with their barbed ends toward the tips of the microvilli (38). Therefore, these myosins would only transport membrane proteins toward the microvillar tip.

A flow model has been proposed that integrates barbed-end myosin movement into models for receptor transport into the coated pits (25). In this model, myosins transport components up the microvillus, but because the microvillar surface is not expandable, the upward migration of the membrane-embedded proteins results in a downward displacement of other membrane components (e.g., ligand-bound receptors) down the microvillus. This model assumes there is a constant flow of receptors, ligand bound or otherwise, a feature that has not been documented. In addition, this model does not allow for regulated movement of ligand-bound receptors into the clathrin-coated regions. Regulated movement down the microvilli would require a pointed-end-directed myosin motor to bind and transport components directly down the microvillus. Only recently has such a pointed-end-directed myosin been identified, an unconventional myosin called myosin VI (45).

The first vertebrate myosin VI was cloned from the porcine kidney cell line LLC-PK₁ (27). Although ubiquitously expressed, myosin VI is most highly expressed in the kidney proximal tubule, where it is localized to the apical domain (27). Myosin VI was shown to be a pointed-end-directed motor that used a baculovirus-expressed truncated containing the catalytic motor domain (45). In keeping with this directionality of movement, myosin VI is present at the base of microvilli in the intestine and at the base of stereocilia in inner ear sensory hair cells (26, 28), suggesting that myosin VI transports components down these actin projections.

In this study, we investigated myosin VI in proximal tubule cells. We found that myosin VI is enriched in the clathrin-rich region IMV microdomain of the brush border, although a small fraction of myosin VI is evident in microvilli. Myosin VI targeting to the developing brush border occurs developmentally at a time commensurate with the beginning of glomerular filtration, which implicates myosin VI in endocytosis. These results suggest that myosin VI may well be involved in the transport of receptors from the microvillus into the clathrin-coated pits during the process of receptor-mediated endocytosis.

METHODS

Primary antibodies. Affinity-purified rabbit anti-myosin VI antibody was raised to the COOH-terminal tail of myosin VI and used as described (27). A monoclonal antibody to adapter protein (AP)-2 was purchased as ascites fluid from Affinity Bioreagents (Golden, CO). An affinity-purified monoclonal antibody to villin was purchased from Amac (Westbrook, ME). A fluorescein-conjugated goat IgG fraction α-horseradish peroxidase (α-HRP) was purchased from ICN Pharmaceuticals (Aurora, OH).

Antibody conjugates. For indirect immunofluorescence microscopy, Alexa Fluor 594-conjugated goat anti-mouse and Alexa Fluor 488-conjugated goat anti-rabbit antibodies were purchased from Molecular Probes (Eugene, OR). For immunoblotting, HRP-conjugated goat anti-mouse (γ-chain specific) and goat anti-rabbit (heavy- and light-chain specific) antibodies were purchased from Zymed Laboratories (San Francisco, CA). For immunoelectron microscopy, 10 nm of gold coated with goat anti-rabbit IgG were purchased from Goldmark Biologicals (Phillipsburg, NJ).

Tissue preparation for electron microscopy and immunocytochemistry. For studies using semithin cryosections of fixed tissue, cryosections were etched Epon sections, adult and neonatal rats (Charles River) were anesthetized intraperitoneally with pentobarbital sodium. The kidneys were perfusion fixed with paraformaldehyde-lysine-periodate (PLP) fixative (37) or with 2% paraformaldehyde as described previously (4). Blocks of fixed kidney were postfixed in the same fixative for an additional 2–4 h.

For both immunofluorescence labeling of semithin cryosections and immunogold labeling of ultrathin cryosections, tissue was cryoprotected by incubating for 1 h in 2.3 M sucrose with 50% polyvinylpyrrolidone, mounted on aluminum nails, and stored in liquid nitrogen (4). Semithin cryosections (0.5 μm) were cut with a Reichert Ultracut E ultramicrotome fitted with an FC-4E cryoattachment. Sections were mounted on Superfrost Plus glass slides (Electron Microscopy Sciences, Fort Washington, PA), and the sections were stained as described previously (4, 5). For double-label experiments, pilot studies were performed to ensure that the fluorochrome-conjugated secondary antibodies did not cross-react with the inappropriate primary antibody. The stained sections were examined and photographed with a Zeiss Axiopt microscope. In double-label experiments, combined images were photographed through a fluorescein/tetramethylrhodamine dye combination filter (Molecular Probes).

Antigen retrieval for immunocytochemistry. In some experiments, antigen retrieval was needed to achieve staining with specific antibodies. In this study, two methods were used. For some antibodies (i.e., staining for AP-2 and myosin VI; see Fig. 4), cryosections were preincubated with 1% SDS in PBS for 5 min before being labeled as described above. In other experiments (Figs. 1B; see Fig. 7) etching of Epon-embedded tissue was utilized. Here, fixed tissue was embedded in Epon 812 as described previously (4), except that the tissue was not subjected to osmium tetroxide or uranyl acetate steps. After being embedded, 0.5-μm sections were cut with glass knives, and the sections were mounted on glass slides. The sections were then etched by incubating for 5 min in a solution containing 10 ml of 100% methanol, 5 ml of propylene oxide, and 2 g of KOH. The slides were then washed two times for 5 min each in 100% methanol and once in Tris-buffered saline (TBS; 50 mM Tris-HCl, 100 mM NaCl, pH 7.4). For antigen retrieval, a 10 mM sodium citrate buffer (pH 6.0) was used. Briefly, 500 ml of buffer in a 2-liter glass beaker were heated to boiling in a microwave oven. The slides were added to the hot buffer and heated in the microwave oven for 20 min at ~40% power. After cooling, the sections were washed three times for 15 min each in TBS, quenched for 15 min in 0.5 M ammonium chloride, and...
injected, the kidneys were perfusion cardiac puncture. Five to ten minutes after the tracer was injected into the easily accessible mesenteric vein. For labeling adult rats, after an abdominal incision, HRP (25 mg; Sigma) in PBS (1 ml) was injected with pentobarbitol sodium. For labeling neonatal rats, 50–100 µl of HRP in PBS was injected via cardiac puncture. The etching procedure unmasks myosin VI epitopes in the microvilli (small arrows), although the majority of the myosin VI staining is in the intermicrovillar (IMV) region (large arrows). Bar, 10 µm.

Fig. 1. Localization of myosin VI in the proximal tubule of the adult rat kidney by indirect immunofluorescence microscopy. A: semithin (0.5-µm) cryosection of paraformaldehyde-lysine-periodeate fixative (PLP)-fixed adult rat kidney was stained with antibodies to myosin VI (green). The location of myosin VI (small arrows) is double exposed with the phase contrast image of the same section. Note that, with this technique, the microvilli of the brush border (large arrows) are not stained. B: etched Epon section of PLP-fixed adult rat kidney stained for myosin VI as in A. The etching procedure unmasks myosin VI epitopes in the microvilli (small arrows), although the majority of the myosin VI staining is in the intermicrovillar (IMV) region (large arrows). Bar, 10 µm.

For indirect immunofluorescence labeling of 5-µm frozen sections of formaldehyde-fixed rat kidney (Fig. 2), all methods were undertaken as described (26, 27). Labeled samples were viewed on a Bio-Rad MRC1024 confocal microscope equipped with an ultraviolet laser. Digital images were compiled in Adobe Photoshop.

For immunoelectron microscopy of ultrathin cryosections, sections were cut with a Reichert Ultracut E ultramicrotome fitted with an FC-4E cryoattachment and picked up on carbon-coated Formvar grids. Sections were labeled overnight with affinity-purified rabbit anti-myosin VI diluted 1:50 in TBS containing 0.1% BSA and 10% goat serum. After being washed with TBS with 0.1% BSA, the sections were incubated for 1 h in the goat anti-rabbit gold conjugate, which was diluted 1:20 in TBS containing 0.1% BSA and 10% goat serum. After being processed as described previously (3, 4), the sections were examined and photographed with a Zeiss 910 electron microscope. Digital images were prepared by using Adobe Photoshop.

Labeling endosomes in the proximal tubule with HRP. Renal endosomes were labeled in vivo with HRP as follows. Sprague-Dawley rats were intraperitoneally anesthetized with pentobarbital sodium. For labeling adult rats, after an abdominal incision, HRP (25 mg; Sigma) in PBS (1 ml) was injected into the easily accessible mesenteric vein. For labeling neonatal rats, 50–100 µl of HRP in PBS was injected via cardiac puncture. Five to ten minutes after the tracer was injected, the kidneys were perfusion fixed with PLP as described above.

Preparation of renal membrane fractions by using Opti-Prep density gradients. Adult male New Zealand White rabbits (Gabrielle Farms, Woodstock, CT) were killed by injection of pentobarbital sodium (Butler, Columbus, OH). Postmitochondrial microsomes were prepared from the renal cortex precisely as described (2). Tissue from one kidney was homogenized in 35 ml of homogenization buffer [Tricine (20 mM; pH 7.8), 8% sucrose and pepstatin A (0.7 µg/ml), leupeptin (0.5 ug/ml), phenylmethylsulfonyl fluoride (40 µg/ml), and EDTA (1 mM) protease inhibitors]. The homogenate was subjected to differential centrifugation as described previously (2). The membrane pellet obtained after the 48,000-g spin was resuspended in 5% OptiPrep (Nycomed Pharma, Oslo, Norway) at a concentration of 10–20 mg/ml. Protein concentrations were determined by the method of Lowry (36). Postmitochondrial microsomes in 5% OptiPrep (1–5 mg) were layered on top of preformed 15–25% OptiPrep gradients. Gradients were centrifuged to equilibrium (2–3 h) at 28,000 rpm by using an SW 41 rotor. Fractions (1 ml) were manually collected from the top and stored at −70°C. For analysis by immunoblotting, equal volumes of each fraction were used.

SDS-PAGE and immunoblotting. Protein samples were solubilized in SDS-PAGE sample buffer and separated by SDS-PAGE using 7.5% polyacrylamide gels according to Laemml (33). For immunoblotting, proteins were transferred to polyvinylidene difluoride and incubated with antibodies as previously described (2). Bound antibody was detected with an enhanced chemiluminescence system (Amersham, Arlington Heights, IL) according to the manufacturer’s protocols.

RESULTS

Myosin VI differentially localizes to microdomains of the brush border in proximal tubule cells of the adult rat kidney. In agreement with earlier studies (27) when we surveyed the rat kidney at low magnification by immunocytochemistry, we found that myosin VI was only expressed in the brush border of the proximal tubule (data not shown). The renal brush border is
morphologically complex and contains at least two distinct microdomains that are structurally and biochemically unique (40). The microvilli consist of villin-rich, actin-based cores and associated plasma membrane that serve to amplify the apical surface of the proximal tubule cells. Between the microvilli, the plasma membrane invaginates into the cytoplasm, forming the IMV clefts or coated-pit region with its distinctive clathrin coat (40). The IMV, enriched in receptors such as megalin, represents the beginning of the endocytic pathway in these cells. By using varied fixation and staining procedures that allowed for high-resolution imaging of these microdomains, we consistently found that myosin VI was enriched in the IMV region (see Figs. 1, 2, and 4). With all staining conditions, myosin VI was never detected within the Golgi complex, as has been suggested by other laboratories (11).

Figure 1A shows the localization of myosin VI in the proximal tubule of a semithin cryosection of PLP-fixed kidney. Here, staining for myosin VI is restricted to the IMV at the base of the brush border. However, after antigen retrieval (Fig. 1B), which seemed to increase the sensitivity of our labeling, staining for myosin VI was also seen on the microvilli. These data show that, although most of the myosin VI found in the proximal tubule is concentrated in or near the IMV, a small but significant pool of myosin VI is also expressed on the microvilli.

The enrichment of myosin VI at the base of the brush border can be further appreciated in Fig. 2. Here, we used double labeling to localize both myosin VI and F-actin. In these experiments, the actin-rich microvilli are strongly stained for F-actin. Myosin VI was positioned at the F-actin rootlets and was excluded from cortical actin-rich regions and cell-cell junctions.

Myosin VI is enriched in the early portion of the endocytic pathway in proximal tubules. The endocytic pathway in the proximal tubule is similar to that described in other cells and consists of clathrin-coated pits and vesicles, uncoated trafficking vesicles, and large endocytic vacuoles (LEV) (15). From the LEV, receptors traffic back to the plasma membrane, whereas their ligands are transported by vesicular carriers to lysosomes. To more accurately evaluate where myosin VI associates along the endocytic pathway, we compared the location of myosin VI with that of an endocytosed tracer in the proximal tubule. As shown in Fig. 3, 10 min after an intravenous injection of HRP, the tracer was found concentrated in the LEV in the apical cytoplasm of proximal tubules. In addition, this figure shows that, in sections that were cut parallel to the apical-basolateral axis of the cell, myosin VI was never observed as associated with the HRP-labeled endosomes.

Most of the staining for myosin VI was found within a region of the proximal tubule bounded by the basal aspect of the microvilli and the HRP-labeled endosomes. Within this region lies the IMV of the plasma membrane as well as numerous trafficking vesicles. To further evaluate the location of myosin VI within this region of the cell, we compared it with that of AP-2, a prominent component of clathrin-coated pits at the plasma membrane. As shown in Fig. 4, myosin VI colocalizes extensively with AP-2 at the base of the brush border in proximal tubule cells, placing myosin VI at or very close to the clathrin-coated pits. However, in clathrin-coated regions that extended deep into the cytoplasm (Fig. 4C, arrows), distinct staining for AP-2 was seen that had little or no staining for myosin VI. This observation suggested potential associations for myosin VI with the bases of microvilli at the top of the IMV invagination.

Finally, we used immunoelectron microscopy to further confirm the subcellular location of myosin VI in this domain. As shown in Fig. 5, consistent with our immunofluorescence data, immunogold staining for...
myosin VI was restricted to the cytoplasm adjacent to the surface of the IMV region of the brush border. We also noted myosin VI around the vesicles that are closely associated with this region of the cell, and gold label was frequently seen along the membrane of IMV invaginations (Fig. 5, B and C). Also consistent with our immunofluorescence data, secondary endosomes were not labeled for myosin VI. Light staining was also detected in the core of microvilli supporting the immunofluorescence staining that we reported after the antigen retrieval. Together, these data show that, in the adult kidney, myosin VI is enriched in the IMV domain in a manner consistent with a role in the early steps of endocytosis.

**Myosin VI cosediments in density gradients with the IMV membranes of the brush border.** Our data suggested that myosin VI interacted with membrane lipids or proteins that were localized to the coated-pit IMV region of the brush border. To test this idea, we compared the density of myosin VI with that of IMV by using density gradients. We have recently shown that the IMV microdomain, characterized by its enrichment in receptors such as megalin, can be separated from microvilli and endosomes by using isopycnic centrifugation combined with continuous OptiPrep density gradients (2). We separated rabbit kidney cortical microsomes on density gradients and analyzed the fractions by immunoblotting. We probed for myosin VI and compared its profile to that seen for the microvillar protein villin and the clathrin AP-2. As shown in Fig. 6, the myosin VI protein was enriched within the same dense membrane fractions that contained the clathrin-coated-pit marker AP-2. These results are consistent with our previous studies that showed that clathrin was enriched in these dense fractions (2). Because myosin VI cosediments with the IMV-enriched mem-

Fig. 3. Comparison of myosin VI to the endocytic tracer horseradish peroxidase (HRP) in the proximal tubule. HRP was injected intravenously into rats. After 10 min, the kidneys were perfusion fixed with PLP, and the tissue was prepared for immunocytochemistry. Semithin cryosections were double labeled with antibodies for HRP (green) and for myosin VI (red). The staining for myosin VI (white arrowheads) is distinct from that seen for HRP in large endocytic vacuoles (black arrowheads).

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Fig. 4. Myosin VI and clathrin adapter protein (AP)-2 colocalize in the proximal tubule. Semithin cryosections of PLP-fixed rat kidney were double labeled for myosin VI (green; A) and for AP-2 (red; B). C: double image of myosin VI and AP-2 staining. Overlap between the 2 proteins is seen as yellow. Arrows, areas of AP-2 staining where there is no myosin VI. Bars, 10 μm.
branes, myosin VI must interact directly by means of other membrane components.

Expression of myosin VI in the developing proximal tubule correlates with the onset of endocytosis. In the neonatal kidney, the onset of endocytosis in the proximal tubule begins during stage III of development and is closely related to the development of distinct microdomains (microvilli and coated pits) within the brush border (4) and to the onset of glomerular filtration. Concomitant with this process, the brush-border receptors such as megalin (4) and transporters such as the type 3 Na\(^+\)/H\(^+\) exchange transporter (5) are shown to redistribute to the apical domain. We speculated that, if myosin VI were involved in endocytic activity in proximal tubule cells, myosin VI would similarly exhibit a developmentally regulated expression profile.

Immunolocalization studies of myosin VI were undertaken in the 1-day-old rat kidney. Animals of this age are still undergoing kidney development, and all stages (35) can be visualized in sections from the same animal. As shown in Fig. 7A, myosin VI expression was restricted to the proximal tubule. Myosin VI was first detected in the S-shaped bodies (Fig. 7B). Here, myosin VI exhibited a diffuse cytoplasmic stain and was restricted to the portion that was destined to become the proximal tubule. Glomerular staining was not seen. In stage III, myosin VI began to be enriched in the apical domain (Fig. 7, B and C), although cytoplasmic stain-
ing was still evident. By stage IV, myosin VI was fully redistributed to the apical domain of the cell. We confirmed that filtration had initiated in stage IV by perfusing the animals with HRP before the kidneys were prepared. As shown in Fig. 7D, only stage IV tubules were capable of reabsorbing the HRP tracer. As we had previously shown, myosin VI did not colocalize with the HRP-staining endosomes. However, in all tubules that had taken up the tracer, myosin VI had redistributed to a fully apical location. These results suggest that myosin VI targeting is developmentally regulated and that expression may be linked to the onset of endocytosis.

**DISCUSSION**

By using high-resolution immunocytochemistry, we show here that an unconventional myosin, myosin VI, is prominently expressed along the IMV microdomain of the brush border of the proximal tubule. Furthermore, membrane fractionation experiments showed interaction of myosin VI with these membranes. We also show that, during development, the expression of myosin VI closely parallels the onset of glomerular filtration, the maturation of the brush border, and the onset of protein reabsorption within the proximal tubule. Together these data suggest a role for myosin VI in some aspect of the endocytic process of this segment.

In the kidney, reabsorption of protein from the forming urine takes place in the proximal tubule and is accomplished primarily by receptor-mediated endocytosis across the apical (brush-border) membrane. This process requires clustering of receptors into clathrin-coated pits at the base of the brush border into the IMV. Although the bulk of the myosin VI in the proximal tubule is associated with the IMV domain, we also found a small but significant population of myosin VI in the microvilli. Myosin VI may be the motor responsible for targeting ligand-bound receptors to the clathrin-coated pits. In support of this idea is the fact that myosin VI is directed toward the pointed end of F-actin. Because the actin cores of microvilli have their pointed ends at the bottom toward the IMV domain, the motor properties of myosin VI would be perfectly suited for movement of cargo in this direction.

**Linking myosin VI to brush-border membrane proteins.** Immunocytochemical studies have shown that several brush-border receptors and transporters are clustered into the IMV region. These proteins include a specific oligomeric form of the type 3 Na\(^+/\)H\(^+\) exchange transporter (2), the folate-binding protein (30), the scavenger receptor megalin (4, 16, 32), the gp280 intrinsic factor-vitamin B\(_12\) receptor (14, 41, 42), and the insulin-like growth factor II/mannose-6-phosphate receptor (19). Past studies have found no conserved mechanism to target these membrane components into the IMV domain. We suggest that the association of myosin VI, directly or by means of some type of adapter protein, may facilitate this process.

One candidate for such an adapter protein is the PSD-95/Dlg/ZO-1 domain containing interacting protein, COOH terminus (GIPC)-M-SemF cytoplasmic domain-associated protein (SEMCAP-1). Myosin VI was identified as a binding partner of GIPC-SEMCAP-1 in a yeast two-hybrid screen (9). The PSD-95/Dlg/ZO-1 domain of GIPC-SEMCAP-1 has been shown to bind to myriad membrane proteins and plasma membrane receptors as judged by the yeast two-hybrid screen method, a list that includes the scavenger receptor megalin (24). Our preliminary studies place GIPC-SEMCAP-1 in the proximal tubule brush border (Hasson T, unpublished observations), and immunolocalization studies have placed GIPC-SEMCAP-1 in clathrin-coated pits (21). We are now in the process of assessing whether GIPC-SEMCAP-1 indeed links megalin to myosin VI in the IMV domain.

A splice form of myosin VI is linked to clathrin-coated pits. During the assembly of this manuscript, Buss et al. (10) reported that a novel, longer splice form of myosin VI colocalized with clathrin-coated pits when expressed in cultured cells. PCR analysis suggested that it was this longer splice form of myosin VI that was the major form expressed in rat kidney. We have not evaluated the myosin VI isoform expressed in our rabbit or rat kidney samples, but the results of Buss et al. are consistent with our observations. Buss et al. further reported that a fraction of the myosin VI could be coimmunoprecipitated with antibodies to AP-2 or clathrin (10). Although we have not shown direct interaction of myosin VI with AP-2 or clathrin in the kidney, our membrane fractionation data showing that...
myosin VI associates with dense membranes that are enriched in AP-2 are consistent with this study. Finally, Buss et al. reported that myosin VI was enriched in clathrin-coated vesicles isolated from calf brain, even though the longer myosin VI splice form was not observed to be expressed in brain (10). Therefore, regardless of the splice form present, all isoforms of myosin VI have the potential to associate with clath-

Fig. 7. Myosin VI expression during renal development. Kidneys from 1-day-old rats, some of which had been injected with HRP (see METHODS), were perfusion fixed and embedded in Epon. Sections (0.5 μm) were etched and stained for myosin VI (A–C) or double labeled for HRP and myosin VI (D). A: low-magnification image showing the renal capsule (arrows) and the nephrogenic zone immediately below. Myosin VI (red) is restricted to proximal tubules. B: staining of a very early stage II capillary loop nephron. Myosin VI (green) begins abruptly (arrows) at the beginning of the proximal segment. The glomerular portion (G) is unstained. Note that this image is overexposed to better show the cytoplasmic staining in the developing proximal tubule. In the stage III tubules seen in D, myosin VI is concentrated at the apical membrane. C: staining of a stage IV proximal tubule (*) reveals that myosin VI (green) is targeted to the apical domain, whereas in earlier stages (arrowheads) myosin VI exhibits a prominent cytoplasmic stain with only minor apical enrichment. D: comparison of the expression of myosin VI (red) with the capacity of the proximal tubules to reabsorb the tracer HRP (green). In stage IV tubules, myosin VI exhibits an apical location distinct from the HRP. Note that the early stage III proximal tubules (arrowheads) did not take up the tracer, and the apical stain is less evident. S, S-shaped body. Bars, 100 μm (A), 10 μm (B–D).
Myosin VI as a general membrane transport motor. Myosin VI is a ubiquitously expressed protein. Highest levels of expression have been seen in polarized epithelial cells, and in these cells, myosin VI is found primarily at the base of microvilli or other actin-based protrusions (26, 28). Therefore, myosin VI may serve a role in endocytic events in tissues other than kidney. Analysis of myosin VI mutants supports this idea. In *Drosophila melanogaster*, myosin VI mutations are responsible for the *jaguar* phenotype (20, 29). The *jaguar* mutation, which is found in the promoter region of the myosin VI gene, affects gene expression only in the testes. *Jaguar* flies have a defect in the individualization stage of spermatogenesis; during this stage, membranes are laid down between each spermatid, separating it from its neighbor. A cone of actin precedes the addition of membrane, and myosin VI is enriched at the leading edge of this cone (29). The mechanism whereby membrane is added between spermatids is unknown, but on the basis of the directionality of myosin VI, two models for myosin VI function in this process have been proposed (18). Both models suggest that myosin VI is associated with membranes. In one model, the motor pulls membrane or membrane components downward along the actin filaments (18). In the other model, myosin VI pulls membrane vesicles inward so that they are in place for fusion to create a new plasma membrane region (18). Both models are consistent with our observations for myosin VI in kidney.

A similar model for myosin VI function has been proposed in the inner ear of the mouse. Myosin VI is expressed exclusively by the sensory hair cells of the inner ear, where it is found at the base of actin projections termed stereocilia (26). Mice with mutations in the myosin VI gene, termed *Snell’s waltzer* (*sv*), are profoundly deaf and exhibit balance dysfunction due to defects in the hair cells (1). Analysis of *sv* mice during development revealed that the stereocilia do not develop properly (43). As the stereocilia develop, the plasma membrane between each projection does not maintain its position at the base of the stereocilium and instead appears to rise up between adjacent stereocilia (43). Ultimately, the stereocilia appear to fuse into large agglomerates that are nonfunctional, resulting in an animal that is deaf and cannot balance. Based again on the directionality of myosin VI, it has been proposed that myosin VI may move the membranes down between stereocilia, effectively tethering the membrane to the actin cytoskeleton (43).

This model is in agreement with our observations in the kidney but does not necessarily agree with the immunolocalization data seen for myosin VI in the inner ear. Unlike the cytoskeleton in the kidney, in the inner ear there is an actin meshwork, the cuticular plate, underneath the actin projections. Myosin VI is highly expressed in this actin meshwork and does not exhibit a membrane-associated localization in an IMV zone such as we have seen in the kidney (26). Myosin VI is enriched in an endocytic domain, the pericellular necklace, found in the apical region of the cells (26), but studies of hair cell fluid phase uptake in *sv* mice suggest that unregulated endocytosis in these animals is normal (43). Therefore, myosin VI may have regulated functions in endocytosis, or it may have other functions distinct from those we have proposed in membrane trafficking. For example, the protein may serve a role in actin dynamics, in tethering actin filaments (such as microvilli or stereocilia) into actin meshworks, or in regulating the structure of actin meshworks such as the cuticular plate (18).

In conclusion, our study shows that the expression of myosin VI in the proximal tubule is restricted to a very specific microdomain of the brush border. These data suggest that myosin VI plays a role in the early events of the process of protein reabsorption in this segment of the nephron. Molecular studies of myosin VI and the proteins through which it interacts with the plasma membrane will be important for understanding its role in the function of the proximal tubule.

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