Targeting of membrane transporters in renal epithelia: when cell biology meets physiology

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Brown, Dennis. Targeting of membrane transporters in renal epithelia: when cell biology meets physiology. Am. J. Physiol. Renal Physiol. 278: F192–F201, 2000.—Epithelial cells in the kidney have highly specialized transport mechanisms that differ among the many tubule segments, and among the different cell types that are present in some regions. The purpose of this brief review is to examine some of the major intracellular mechanisms by which the membrane proteins that participate in these differentiated cellular functions are addressed, sorted, and delivered to specific membrane domains of epithelial cells. Unraveling these processes is important not only for our understanding of normal cellular function but is also critical for the interpretation of pathophysiological dysfunction in the context of newly generated molecular and cellular information concerning hereditary and acquired transporter abnormalities. Among the topics covered are sorting signals on proteins, role of the cytoskeleton, vesicle coat proteins, the fusion machinery, and exo- and endocytosis of recycling proteins. Examples of these events in renal epithelial cells are highlighted throughout this review and are related to the physiology of the kidney.

protein sorting; epithelial polarity; cytoskeleton; vesicle trafficking; membrane recycling

THROUGHOUT THE LAST DECADE, dramatic progress has been made in identifying many membrane-associated proteins that participate in the multitude of complex, vectorial transport processes that are essential for kidney function. These include, but are not limited to, aquaporins, sodium-hydrogen exchangers, urea transporters, amino acid transporters, glucose transporters, anion exchangers, thiazide and bumetanide-sensitive NaCl and Na+-K+-2Cl- cotransporters, chloride channels, etc. In addition, a host of other important membrane proteins, including receptors, enzymes, adhesion molecules, and junctional proteins, have also been identified and localized in renal epithelial cells (20). These discoveries have been paralleled by the identification of human diseases that are linked to mutations in the genes encoding a variety of epithelial cell membrane proteins, including CLC5 and the cystic fibrosis transmembrane conductance regulator chloride channels (32, 64), aquaporins (37), H+-ATPase subunits (66), and sodium channels (25, 54). In some cases, the activity of the protein is directly affected by the mutation, whereas in other cases the mutation results in a failure of the intracellular machinery to deliver an otherwise functional protein to its correct location within the epithelial cell. These latter pathophysiologies can be grouped together as "diseases of protein sorting." In addition, some kidney diseases result from acquired defects. These include lithium-induced diuresis resulting from downregulation of aquaporin-2 (AQP2) (78) and cadmium-induced Fanconi-like syndrome, the latter of which results from defective apical membrane protein recycling in the proximal tubule (59). Because normal epithelial cell function depends on accurate delivery of this vast array of membrane components to particular domains of the cell surface, and because defective targeting can lead to disease, it is crucial to understand how each cell type interprets intrinsic sorting signals that are embedded within the amino acid sequence of each protein. This review briefly considers several of the steps that can be involved in the process of sorting, addressing, and delivering proteins to their target membranes (Fig. 1) and examines how these processes may be related to some aspects of kidney physiology.
Intrinsic Protein Sorting Signals

It has long been realized that specific sorting information is located within the sequence of both transmembrane and cytosolic proteins that determines their ultimate destination within a cell. On the basis of the early observation that different viruses could bud from either the apical (influenza virus) or the basolateral (vesicular stomatitis virus) pole of epithelial cells (43, 93), it was subsequently determined that the viral coat proteins themselves, when transfected alone into cells, also showed a similar polarity of membrane insertion (133). These key data implied that the coat proteins themselves contained targeting information that was sufficient to direct them to a specific membrane domain of the cell. A considerable amount of work has been performed in a variety of systems to dissect protein sequences to identify the nature of their intrinsic targeting information. Among the key findings are the existence of tyrosine-based signals that direct proteins to the basolateral plasma membrane and that also play a role in concentrating proteins into cation-coated pits for endocytosis. These motifs include YXRF in the transferrin receptor (34), and NPXY in the low-density lipoprotein receptor and gp330/megalin (30). Many other membrane proteins contain similar motifs. A COOH-terminal dileucine motif (LL) has also been implicated in directing proteins into the cation-mediated endocytotic pathway, although endocytosis of the GLUT-4 glucose transporter also involves interaction of the COOH terminus with distinct motifs in the NH2 terminus of the protein (35, 121). Other proteins such as the vasopressin V2 receptor also contain a COOH-terminal dileucine motif (9). Finally, basolateral targeting of the polymeric Ig receptor involves a unique 14-amino-acid sequence in the COOH terminus that has not so far been detected on other proteins (28). However, the sorting signal of many other basolateral proteins remains to be identified, suggesting that nonidentical amino acid sequences can have similar properties and contain similar targeting information. In much the same way, nonidentical hydrophobic stretches of amino acids act as signal sequences to direct the translocation of membrane and secreted proteins across the membrane of the rough endoplasmic reticulum (124).

Although it was originally believed that basolateral sorting was the default pathway, and that apical proteins would contain recognizable signals, amino acid-based apical sorting motifs have proven very elusive. However, proteins that are tethered to the lipid bilayer by a glycosylphosphatidylinositol (GPI) anchor rather than by a traditional transmembrane domain are in most (but not all) cases delivered apically (23, 134). GPI-linked proteins are probably sorted into specific, apically directed carrier vesicles at the level of the trans-Golgi network, after interaction with so-called glycolipid rafts in Golgi membranes (55). This may involve an interaction with the protein caveolin, a component of cell-surface pinocytotic invaginations (caveolae) as well as post-Golgi vesicles (96, 109). However, it is noteworthy that caveolae themselves are almost exclusively found on basolateral plasma membrane domains in epithelial cells, an observation that is not compatible with a proposed role in concentrating (mainly apical) GPI-anchored proteins in the plasma membrane (11, 74, 123).

Some proteins, including aquaporin 1, are delivered to both apical and basolateral plasma membranes of epithelial cells, including proximal tubule cells (84, 101) and absorptive cells in the efferent ducts of the male reproductive tract (21). When transfected into LLC-PK1 cells, AQP1 also distributes to both apical and basolateral membranes (68). Whether this distribution reflects the absence of any specific signal, or the presence of a signal that allows insertion into both membrane domains, remains to be established.

One of the first targeting signals to be recognized on any protein was the mannose-6-phosphate residue on lysosomal hydrolases. This posttranslational modification allows these hydrolases to interact with mannose-6-phosphate receptors in the Golgi and to be packaged into vesicles that deliver these hydrolases to lysosomes (24, 113). However, glycosylation per se does not seem to be necessary for the correct targeting of most glycosylated proteins. For example, AQP1 and AQP2 are heavily glycosylated, but absence of glycosylation has little or no effect on either targeting or on water channel function (7, 120). There are scattered reports describing an effect of sugar residues on targeting, but whether this is a widespread requirement remains to be determined (8, 104).

The Cytoskeleton

Microtubules. The role of microtubules in epithelial cell function and protein trafficking is well established (20). Microtubule disruption by colchicine or nocodazole perturbs the delivery of both newly synthesized and recycling proteins to the cell surface and causes a
marked shift in the distribution of many membrane proteins from their usual surface location onto scattered intracellular vesicles (20). This process affects rapidly recycling proteins more than membrane proteins that have a longer residence time at the cell surface (Fig. 2) (18). Endocytosis continues at least during the initial stages of microtubule disruption, but the internalized proteins can no longer be effectively recycled back to the cell surface in the absence of polymerized microtubules. This results in the intracellular accumulation of rapidly recycling proteins such as AQP2 (100), the H⁺-ATPase (19), and gp330/megalin (51). The functional consequences of microtubule disruption on epithelial transport in various tissues include inhibition of vasopressin-induced water permeability (38, 63, 65, 86, 91, 117), inhibition of luminal acidification (115), and inhibition of phosphate transport (39).

Microtubules interact with transport vesicles and other organelles via ATPase motor proteins of the kinesin and dynein family (72, 106). These proteins use the energy of ATP hydrolysis to move organelles along microtubules in a retrograde (dyneins) or anterograde (kinesins) manner, where anterograde represents movement toward the growing or “+” end of the microtubule. Present key areas of research include determining how these motors interact with the transported vesicles, and how the cytoplasmic domains of transported proteins are involved in determining the specificity of this process (4, 110).

The actin cytoskeleton. Actin filaments also play a role in the intracellular movement of vesicles and organelles via accessory proteins such as myosin ATPases (31). It has been proposed that whereas microtubules are responsible for “long-range” transport processes in the cell, actin filaments are involved in the final steps of access of vesicles to the underside of the plasma membrane (4, 41, 94). An additional role of the actin-based cortical cell web might actually be to restrict unregulated access of vesicles to the membrane by forming a physical barrier that impedes vesicle movement. Only after an appropriate stimulus (e.g., a rise in intracellular calcium) that results in activation of actin-severing or remodeling proteins such as gelosolin can the barrier be loosened to permit vesicles to move toward the plasma membrane. This hypothesis is supported by experimental data from several cell types that have demonstrated an increase in exocytosis after experimental or physiological actin depolymerization (46, 56, 57, 85, 118).

Actin also serves as a major element in an intracellular scaffold that is erected around many membrane proteins. One version of this scaffold involves several interacting proteins, including ankyrin and spectrin/fodrin (53, 82). Na⁺-K⁺-ATPase and AE1 (the band 3 anion exchanger) are just two of the membrane proteins that are cross-linked to the actin cytoskeleton in this way (82). This system serves to retain proteins within specific membrane domains. Proteins that are tethered in this fashion are probably not actively recycling.

PDZ-domain binding proteins. A large and increasing number of proteins have been identified as "PDZ-domain" proteins. These proteins, named after the initials of the first family members to be described (PSD-95, a mammalian postsynaptic density protein; the disks large protein of Drosophila; and the tight-
junction protein ZO-1) have long (up to 100) amino acid stretches that form binding pockets for PDZ-binding domains located on other proteins (40). The PDZ-binding domains are usually at the extreme COOH terminus of the protein and include the motif DTAL. PDZ-binding proteins appear to be maintained in specific cell surface domains by interacting with PDZ proteins, which themselves usually possess a binding domain that allows indirect interaction with the actin cytoskeleton. This provides another form of actin-based scaffolding system for maintaining proteins in specific cell-surface domains. Because PDZ proteins contain more than one binding pocket, it is thought that clusters of different but functionally related membrane proteins can be clustered together in an efficient unit by this type of interaction. Many PDZ-binding proteins have been identified, including the cystic fibrosis transmembrane conductance regulator (111, 125), potassium channels (26, 69, 70), the β-adrenergic receptor (52), and nitric oxide synthase (105), to name only a few.

The B1 H\(^+\)-ATPase subunit is a PDZ-binding protein. We have recently found that the 56-kDa B1 subunit of the vacuolar ATPase is a PDZ-binding protein that can associate with the PDZ-protein known as NHE-RF (13). NHE-RF was originally discovered and named because of its interaction with, and regulation of, the NHE3 sodium-hydrogen exchanger in the apical membrane of proximal tubule epithelial cells (128, 129). Our recent data show that NHE-RF is indeed colocalized with NHE3 in the brush-border membrane, but in addition, we found that NHE-RF is colocalized with the H\(^+\)-ATPase in B-intercalated cells of the kidney but is expressed in much lower amounts in the A cell (13). This raises the possibility that the variable phenotype of the B cell, which can have an apical, basolateral, or bipolar H\(^+\)-ATPase distribution (15), is in some way regulated by a PDZ-domain scaffolding interaction via the 56-kDa H\(^+\)-ATPase subunit. Interestingly, we reported previously that the 56-kDa subunit of the H\(^+\)-ATPase was associated with AQP2-containing endosomes in collecting duct principal cells, which did not contain some of the other H\(^+\)-ATPase subunits, including the 16-kDa transmembrane proteolipid subunit c (102). Because the 56-kDa B1 subunit has no transmembrane domain, we postulated that it must be attached to the endosome membrane by interaction with other proteins. Our present data raise the possibility that this association could be via a yet unidentified PDZ-domain protein that is located on these endosomes. It is also intriguing that a different isoform of the 56-kDa subunit, the B2 isoform, is expressed in the proximal tubule and that this isoform has a COOH-terminal truncation that removes the PDZ-binding motif (81). The functional significance of this cell-specific isoform expression remains to be determined.

Vesicle Coat Proteins

Vesicle transport is an integral part of all steps of the biosynthetic and recycling pathways that result in the delivery of proteins to the plasma membrane, and their recovery into the cytoplasm by endocytosis. Some of the vesicles that participate in various parts of the pathway are well defined, whereas others are poorly defined. The most recognizable vesicles are those that have distinct morphological characteristics, usually a “coat” of material on their cytoplasmic surface, detectable by various electron microscopic procedures (Fig. 3).

Clathrin. The first coat protein to be identified was clathrin, a coat protein associated with endocytotic coated pits and vesicles, and with some vesicles that bud from the trans-Golgi network (47, 49, 88). Although the precise role of clathrin is not completely understood, it forms a geometric lattice around the invaginating membrane domain and may be involved in the actual formation of the vesicle, as well as in anchoring specific membrane proteins in the forming vesicle to allow their efficient internalization, via a cross-linking interaction with specific “adaptor” proteins (61). In the kidney, the proximal tubule has a very extensive apical clathrin coat that is responsible for much of the endocytosis of filtered proteins in this nephron segment (92).

The promiscuous receptor gp330/megalin is concentrated in these proximal tubule clathrin-coated pits and plays a major role in the receptor-mediated endocytosis of many filtered ligands (33, 36). In the collecting duct, the internalization step of vasopressin-stimulated AQP2 membrane recycling is believed to occur via apical clathrin-coated pits (17, 22, 116). Although no clear evidence that AQP2 accumulates in coated pits has yet been obtained in the kidney, preliminary studies by fracture labeling on AQP2-transfected LLC-PK1 cells have shown that AQP2 can cluster into structures on the cell surface that resemble coated pits in freeze-fracture replicas (119).

Caveolin. Caveolin was originally identified as a component of post-Golgi vesicles and was named V1P21 (vesicle integral protein of 21 kDa) (71). It was subsequently found in association with caveoleae, which are 60- to 90-nm vesicles or invaginations located at the plasma membrane of many cell types (96). As discussed
earlier, caveolae are membrane domains that may be involved in the accumulation, trafficking, and internalization of a select group of proteins that associate with glycolipid-rich regions of the membrane (87). However, any hypothesis concerning their role must take into account the almost unique occurrence of caveolae on the basolateral surface of epithelial cells, and their scarcity at the apical plasma membrane (11, 123). In the kidney, caveolae are most abundant in distal tubule epithelial cells and in collecting duct principal cells. They appear to be absent from proximal tubules and intercalated cells (11). The calcium ATPase has been localized to caveolae in the distal tubule by immunogold electron microscopy (42). By fractionation methods, a whole host of other membrane-associated proteins, including GTP-binding proteins that are involved in signal transduction, have been found in association with Triton X-100-insoluble membrane domains in which the protein caveolin is also enriched (73, 103). However, other data have shown that caveolae isolated by affinity purification with anticaeven antibodies contain a much more restricted set of proteins (114). Thus the relationship among Triton X-100-insoluble domains, caveolae, and the respective proteins that they contain is still a matter of some dispute.

β-COP and the coatomer complex. A type of non-clathrin-coated vesicle was identified in the Golgi region of cells by electron microscopy and was subsequently found to have a distinct coat formed of several protein subunits that together are called the coatomer complex. One of the first to be identified was β-COP, and other COP components rapidly followed (97). These vesicles are now believed to be involved in intracellular transport of proteins among Golgi cisternae, as well as between the Golgi and the rough endoplasmic reticulum (89). A specific set of coat components has been identified on vesicles budding from the rough endoplasmic reticulum (5). COP proteins have also been found on endosomes in some cells, and their role may be extended to some aspects of endosome function (130). Assembly of the COP coat is believed to be required for vesicle budding to occur, in some as yet unspecified way, and this assembly process requires the activity of ADP-ribosylation factor proteins, and phospholipase D isoforms (95, 108).

The H+-ATPase coat. Vesicles involved in the trafficking of the H+-ATPase to and from the cell surface of intercalated cells, as well as other proton secreting “mitochondria-rich” cells, have a distinct coat that contains the cytoplasmic subunits (the V1 domain) of the enzyme (14). We have shown that these vesicles do not contain clathrin (16), caveolin (11), or β-COP (10), and the conclusion is that they represent a novel class of transport vesicle that is capable of undergoing both exo- and endocytosis by using an as yet unidentified mechanism. However, cellubrevin, which is an analog of the synaptic vesicle protein synaptobrevin, may be involved in the fusion of these vesicles with the plasma membrane, as will be discussed below. Our present hypothesis is that some of the subunits of the V1 domain of the H+-ATPase are involved in the recycling of these vesicles and that they interact with other components of the trafficking and sorting machinery to achieve this end.

The SNARE Hypothesis and Membrane Fusion

Work performed initially on synaptic vesicles and yeast has identified several homologous proteins that associate with plasma membranes and intracellular vesicles to form a so-called fusion machine (27, 98). These proteins interact to allow selective and specific fusion between vesicles and their target membranes to occur. Most fusion events so far examined involve a similar battery of proteins. One group of proteins that participate in this process are called SNAREs [or SNAP receptors, where SNAP is an acronym for soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein]. The SNAREs that are associated with vesicles are known as v-SNAREs, and those on target membranes such as the plasma membrane are t-SNAREs. Membrane-associated SNAREs form a lock and key that is activated by soluble proteins, including NSF, various SNAP isoforms, and small-molecular-weight GTPases (90, 127). In synaptic vesicles, the v-SNARE is known as vesicle-associated membrane protein (VAMP) or synaptobrevin. Cellubrevin is a more ubiquitous homolog found in many nonneuronal cells (79), including proton-secreting mitochondria-rich cells (10). The t-SNAREs include the syntaxin family of proteins, and different syntaxins have different cellular and membrane distributions. Many proteins associated with this fusion machinery have been identified and may play a key role in aquaporin insertion in principal cells of the kidney (58, 76, 77, 83) and in proton-secreting epithelial cells in the kidney and the male reproductive tract (10, 12). Recent data have shown that tetanus toxin, which cleaves cellubrevin and synaptobrevin, inhibits apical proton secretion in inner medullary collecting duct cells in culture (3) and in isolated vas deferens from the reproductive tract (12). The proposed mechanism is via an inhibition of cellubrevin-dependent exocytosis of H+-ATPase-containing vesicles, leading to a progressive loss of plasma membrane H+-ATPase in these cells.

However, although SNARE proteins have a role to play in membrane fusion events, the participation of other components is also essential. In fact, the precise role of the SNARE proteins is very controversial at the moment. A recent study showing that v-SNAREs and t-SNAREs are promiscuous, and do not pair in precise patterns, implies that these proteins are probably not responsible for the specificity of membrane fusion events (131). It is now appreciated that small-molecular-weight G proteins of the Rab family and so-called “tethering proteins” interact to bind membranes together before the input from SNARE proteins (126) and may be more involved in determining specificity (48). These tethering factors are large proteins, such as giantin and early endosome antigen 1 (EEA1), and their tethering function requires Rab activity. For example, endosome-endosome fusion requires EEA1 and the GTP-bound form of rab 5 under normal condi-
tions (112). Furthermore, tethering proteins themselves interact with a variety of additional proteins that complicate the picture even more. The reader is referred to recent reviews for a more detailed description of these issues (48, 126). Thus the emerging picture is that the specificity of membrane targeting requires the sequential interaction of tethering factors, rab GTPases, and SNAREs.

Membrane Recycling

Many membrane proteins are rapidly recycled between intracellular vesicles and the cell surface whereas others have longer residence times at the cell surface. Protein recycling has important implications for epithelial cell function. It allows surface receptors to internalize their ligands, which may include hormones, nutrients, and toxins. It allows the cell to control the cell-surface expression of proteins via variations in the rate of endocytosis and exocytosis of any given protein. The renal proximal tubule can modulate a variety of apical proteins in this way, including megalin/gp330 (33, 51), phosphate transporters (60), and the H^+-ATPase (2). The collecting duct recycles AQP2 between intracellular vesicles and the cell surface in response to vasopressin (1, 44, 122), whereas recycling and polarized expression of the H^+-ATPase is modulated by systemic acid-base conditions (6, 75, 99, 107). These processes are subject to a complex series of control mechanisms, some of which have been discussed above. They often require some posttranslational modification of the transported protein to be activated. For example, phosphorylation is a key event in AQP2 trafficking (45, 67) and is also required for internalization and transcytosis of the polymeric Ig receptor in epithelial cells (29). The recycling of proteins can be interrupted by interventions that neutralize intracellular acidic compartments (80). We have shown that bafilomycin inhibits AQP2 recycling in transfected LLC-PK1 cells and causes an extensive accumulation of AQP2 in the trans-Golgi region of the cell (50). A similar inhibition of AQP2 recycling is caused by low temperature (50). Protein recycling is also inhibited by microtubule disruption (20). These processes are illustrated for AQP2 in Fig. 4.

Why some cellular transport functions are modulated by the insertion and removal of cell surface transporters, and others by “gating” transporters that are already present at the cell surface, is not entirely clear. Presumably, different physiological control mechanisms and appropriate response times are best suited to one mechanism vs. another. However, these two processes are not mutually exclusive, and control of vectorial transport can be achieved both by increased or decreased transporter expression at the plasma membrane as well as by rapid activation/inhibition of cell-surface proteins. It should also be noted that the cellular content of some transporters is chronically regulated at the transcriptional level, leading to increased protein expression. For example, AQP2 mRNA transcription is increased by elevated cAMP via a cAMP-responsive element in the promoter region of the gene (62, 132). Thus both acute and chronic responses to vasopressin regulate cellular and membrane AQP2 expression.

In summary, it is clear that many of the physiological transport processes in the kidney and other transporting epithelia that were elegantly dissected by Carl Gottschalk and his peers can now be at least partially explained at the cell and molecular biological level. Nonetheless, considerable work remains to relate the physiological and pathophysiological regulation and dysregulation of these processes to our present knowl-

![Fig. 4. Diagram showing how some of trafficking steps described in this review may be related to vasopressin (VP)-stimulated insertion of AQP2 in collecting duct principal cells. After binding to its basolateral receptor, VP causes an increase in intracellular cAMP via its action on adenylate cyclase via the heterotrimeric G protein Gs. AQP2 is phosphorylated by protein kinase A (PKA) and is moved toward cell surface in a microtubule- and microfilament-dependent fashion, with involvement of microtubule motors and actin motors. The mechanism by which PKA-dependent phosphorylation of AQP2 results in its appearance at the cell surface is not known, but one possibility is that this allows more efficient interaction with some of the components of transporting and targeting machinery, e.g., microtubules or microfilaments (reproduced from D. Brown, T. Katsura, and C. E. Gustafson. Am. J. Physiol. Renal Physiol. 275: F328–F331, 1998).](http://ajprenal.physiology.org/DownloadedFrom/10.220.32.246)
edge of transporter structure, function, and intracellular trafficking. It should be expected that mutations in genes encoding “generic” accessory proteins that are involved in protein trafficking and targeting might affect many processes and would be incompatible with cell viability. In contrast, mutations in specific transporter genes are already known to cause disease, and the number of such pathophysiological conditions related to transporter malfunction or missorting (either hereditary or acquired) will certainly increase over the next few years. Ultimately, this knowledge must be applied to the whole-animal level to understand the complex interplay among the multitude of physiological stimuli that affect renal function.

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