

# TRANSPORT PROTEIN TRAFFICKING IN POLARIZED CELLS

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■ **Abstract** In order to carry out their physiological functions, ion transport proteins must be targeted to the appropriate domains of cell membranes. Regulation of ion transport activity frequently involves the tightly controlled delivery of intracellular populations of transport proteins to the plasma membrane or the endocytic retrieval of transport proteins from the cell surface. Transport proteins carry signals embedded within their structures that specify their subcellular distributions and endow them with the capacity to participate in regulated membrane trafficking processes. Recently, a great deal has been learned about the biochemical nature of these signals, as well as about the cellular machinery that interprets them and acts upon their messages.

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## INTRODUCTION

Although seemingly straight-forward and self-explanatory, the term membrane transport is, in actuality, deceptively ambiguous. Practitioners of closely related disciplines equip the phrase with different implied prepositions, and thus succeed in drawing from it widely disparate meanings. For cell biologists, membrane transport suggests the transport of membranes, encompassing all the processes associated with the trafficking of membrane-delimited vesicles and their cargoes. In contrast, for cell physiologists, transport by membranes is inferred. In this context, the term relates to the movement of fluid and solute across a lipid bilayer. Both interpretations are, of course, equally correct, and recent progress in both fields is proving them to be ever more intimately intertwined.

Transport of ions and other hydrophilic solutes across hydrophobic lipid membranes requires the participation of specific transport proteins. This enormously large and diverse class of polypeptides, which likely accounts for up to several percent of the human genome, can be divided into a few sub-categories whose members are defined by shared mechanistic and kinetic characteristics. Channels and carriers are permeation pores that provide pathways for diffusion across the bilayer. Although many channels are highly selective and subject to rapid control by gating processes that modulate their accessibility, channels do not interact strongly with the substances that pass through them. Carrier-mediated transport requires the transported substance to bind to a specific and saturable binding site. Net flux of a substance through a channel or a carrier is entirely passive and thus is always downhill with respect to the substance's transmembrane electrochemical gradient. Similar to carriers, co- and counter-transport systems bind directly to their transport substrates. As their name implies, however, co- and counter-transporters mediate the simultaneous movement of at least two different substances in the same or opposite directions. The net flux of both substances is determined by the relative magnitudes of their respective electrochemical gradients. Thus a large gradient favoring the downhill flux of one substance can drive the uphill movement of a co- or counter-transported substance against an unfavorable gradient of smaller absolute magnitude. Finally, pumps bind their transport substrates tightly and couple their transmembrane movement to the hydrolysis of ATP. Consequently, pump-mediated transport can occur against substantial electrochemical gradients. In fact, the activity of pumps is primarily responsible for generating the gradients that are exploited by all of the previously introduced classes of transport proteins.

The design of any transport protein is constrained by the requirement that it forms a hydrophilic pathway through the membrane. Most transport proteins are polytopic, spanning the bilayer anywhere from 2 times, in the case of epithelial

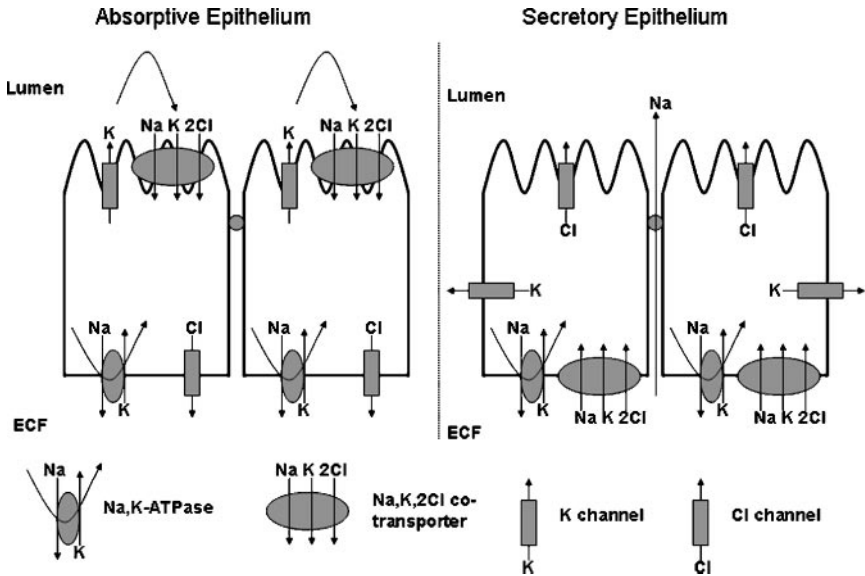
sodium channels (Alvarez de la Rosa et al. 2000), to 24 times in the instance of the voltage-gated sodium channels present in electrically excitable tissues (Catterall 1991). Many transport proteins are composed of homo- or hetero-oligomers of membrane-spanning subunits. Several families of potassium channels, for example, incorporate four identical subunit polypeptides, each of which spans the membrane twice (Aguilar-Bryan et al. 1998). Numerous mechanisms, which are discussed below, have evolved to ensure that these proteins acquire their complex topologies and quaternary structures.

The permeability properties of any biological membrane are determined largely by the inventory of transport proteins it possesses. The membranes defining each sub-cellular organelle are endowed with complements of ion transport proteins that are commensurate with, and often responsible for, specific organelle functions. For example, it is the presence of both an H-ATPase and a Cl channel that accounts for the capacity of endosomes and lysosomes to acidify their lumens (Galloway et al. 1983). Similarly, the endoplasmic reticulum's role in calcium signaling is conferred upon it by the populations of Ca-ATPase and ligand-gated Ca channels (IP<sub>3</sub> and ryanodine receptors) that occupy its membranes (Poulsen et al. 1995, Tuvia et al. 1999).

## EPITHELIAL CELLS

In the case of the plasma membrane, permeability properties are attributable not only to the number and variety but also to the domain-specific distributions of transport proteins. This point is perhaps best illustrated by the example of polarized epithelial cells. The cell surface membranes of polarized epithelial cells are divided into apical and basolateral domains, separated from one another by tight junctions (Caplan 1997b, Nelson & Yeaman 2001). The basolateral domain participates in contacts with the basement membrane and neighboring cells, whereas the apical domain generally confronts a lumen. The asymmetric distribution of transport proteins among these surfaces is responsible for the capacity of epithelia to mediate vectorial transport of solutes or fluids against concentration gradients. Such distribution also determines whether this transport will be secretory or absorptive. A comparison of absorptive and secretory epithelial cells is instructive in this regard (see Figure 1).

The renal epithelial cells that line the thick ascending limb of the nephron's loop of Henle reabsorb Na and Cl from the urine against steep concentration gradients. This is accomplished through a collaboration between the basolateral Na,K-ATPase and a basolateral Cl channel working in series with an apical Na,K,2Cl cotransporter and an apical K channel (Hebert 1998, Lifton et al. 2001). The activity of the sodium pump creates a low intracellular Na concentration, which serves as the driving force for coupled Na,K and Cl entry across the apical membrane. The K that enters via this route leaks back into the urine through the apical K channel. The Na and Cl exit across the basolateral membrane with the help of the Na,K-ATPase and the Cl channel, respectively, resulting in net transepithelial NaCl



**Figure 1** The polarized distributions of ion transporters determine the physiologic properties of transporting epithelia. In the absorptive epithelium that lines the thick ascending limb of Henle's loop in the kidney (*left panel*), the Na,K-ATPase, the Na,K,2Cl cotransporter, a chloride channel, and a potassium channel are distributed among the apical and basolateral plasma membrane domains in a manner that favors the net movement of Na and Cl from the lumen to the extracellular fluid compartment (ECF). In airway and intestinal epithelial cells, the same types of transport proteins are distributed differently to accomplish the secretion of Na and Cl from the ECF to the lumen.

absorption (Figure 1, *left panel*). Airway and intestinal epithelial cells use very similar transport proteins, arrayed in different distributions, to effect net NaCl secretion (Clarke et al. 1992, Flagella et al. 1999, Riordan et al. 1994). In this case, the Na,K-ATPase, K channel and Na,K,2Cl cotransporter are basolateral, whereas the Cl channel is apical (Figure 1, *right panel*). In light of the dramatic influence this transporter distribution exerts over epithelial transport activity, it is tempting to propose that membrane transport, in the cell physiologic sense, is in fact the most significant teleological justification for the existence of epithelial polarity. According to this chauvinistic and untestable hypothesis, the cellular mechanisms responsible for the generation and maintenance of epithelial polarity developed, in large measure, to produce the asymmetrical distribution of transport systems that, in turn, determines an epithelium's contribution to homeostasis.

Clearly, cells must be capable of targeting transport proteins to the appropriate organelle or plasma membrane domains and retaining them there following their delivery. Transport proteins resemble other classes of membrane proteins in possessing sequence motifs that function as sorting signals (Brown & Stow 1996,

Caplan 1997b). These signals are interpreted and acted upon through protein-protein interactions with components of the cellular sorting machinery. Cells can also exploit such interactions in order to control transport activity by regulating the trafficking of transport proteins between intracellular vesicular storage pools and the plasma membrane. Recently, enormous progress has been made in elucidating the sequence motifs and molecular partners that participate in these processes. These advances have provided powerful new insights into the cell biologic pathways cells invoke to modulate their physiologic functions.

## **MECHANISMS AND SIGNALS INVOLVED IN THE GENERATION AND MAINTENANCE OF EPITHELIAL POLARITY**

Over the past 10 to 15 years, several mechanisms used by epithelial cells to establish and maintain the polarized distribution of membrane proteins at the plasmalemma have been identified. One principle that underlies all polarized distribution mechanisms is that sorting information, often in the form of a discrete amino acid sequence, is carried within the structure of the sorted protein. The sorting information is often referred to as a sorting signal, sorting determinant, or trafficking signal. This principle also holds true for protein sorting in neurons (discussed in more detail below). The sorting determinants interact with components located within the cell's cytoplasm that are responsible for delivering the protein to the correct surface domain—these components make up the sorting machinery. The preparations for proper trafficking begin as the surface-destined protein is inserted into the endoplasmic reticulum (ER), continue as the protein passes through the Golgi, and ultimately may be determined by packaging into cargo vesicles budding from the *trans*-Golgi network (TGN). After leaving the TGN protein localization can be regulated further by endocytosis and transcytosis (Mostov et al. 2000). During the journey through the secretory pathway multiple sorting elements within a single protein are required to achieve delivery to the appropriate cell surface or organelle. Upon reaching its destination within the cell, another set of signals are required to maintain the protein's localization or to allow its retrieval from the cell surface in response to physiologic stimuli. There are surprisingly few, if any, common features shared by all of the many classes of sorting signals identified to date.

### **Tyrosine-Based and Di-Leucine Sorting Signals**

One of the most thoroughly studied classes of sorting signal is the tyrosine-based motif (Matter et al. 1992, Matter & Mellman 1994, Thomas et al. 1993). These signals are associated with localization to coated pits and clathrin-mediated endocytosis, but often they are also linked to basolateral sorting (Distel et al. 1998, Hunziker et al. 1991, Lin et al. 1997). These motifs are located in the cytoplasmic

tails of proteins, where they are accessible to interact with sorting machinery such as adaptor complex subunits. The signals contain an essential tyrosine residue and may conform to the canonical NPXY, or YXX $\phi$  (where  $\phi$  is a bulky or hydrophobic residue) formats. Tyrosine-based signals were first identified in the low-density lipoprotein receptor (LDL-R), the polymeric immunoglobulin A receptor (pIgA-R), and the transferrin receptor (Tfr-R) (Matter & Mellman 1994). The fine structure of tyrosine-based motifs has been analyzed, and evidence suggests that many may form a three-dimensional structure known as a tight  $\beta$ -turn (Mostov & Cardone 1995). Whether the  $\beta$ -turn is essential to basolateral targeting and clathrin-mediated endocytosis has yet to be determined. Other critical residues flanking the NPXY/YXX $\phi$  residue motif may determine delivery from the TGN to the basolateral surface or, in other cases, signal for internalization into an endosomal compartment via clathrin-coated pits. The basolateral sorting activity of the signal is essential to establishing the polarity of the protein, whereas the endocytosis signal permits proper surface regulation of the protein. The importance of this regulation has been clearly demonstrated in the case of familial hypercholesterolemia, where mutations to the LDL-R internalization domain prevent the uptake of low-density lipoprotein from the plasma and into liver cells (Chen et al. 1990, Goldstein & Brown 2001).

In addition to tyrosine-based motifs, studies of epithelial protein sorting indicate that a di-leucine motif in the mouse macrophage Fc receptor (FcR2-B2) conveys sorting information (Hunziker & Fumey 1994, Matter et al. 1994). The di-leucine sequence was shown to mediate both basolateral sorting and coated pit localization in the case of FcR2-B2 and has since been found to act in a number of other proteins (Marks et al. 1996). It should be noted that in the case of the human equilibrative nucleoside transporters (hENT2), a di-leucine motif can confer the ability to be regulated by endocytosis without also specifying basolateral sorting (Mangravite et al. 2003). This finding suggests that other protein regions, or amino acid residues, play critical roles in modulating the activities of sorting signals.

## Sorting to the Apical Surface of Epithelia

Delivery of proteins to the apical surfaces of epithelial cells depends on the coordination of multiple, distinct, mechanisms. One of the first significant insights into the nature of apical sorting determinants came from the finding that proteins embedded within the exoplasmic membrane leaflet via a GPI moiety were preferentially sorted to the apical surface (Arreaza et al. 1994, Fiedler et al. 1993, Le Gall et al. 1995, Nosjean et al. 1997). Although subsequent analysis of different cell types and additional GPI-linked proteins showed that this mechanism is not universal (Lipardi et al. 1998, Marzolo et al. 1997, Zurzolo et al. 1993), it did provide impetus for investigators to consider the possibility that domains within the membrane, defined by special lipid content, may be directed preferentially to the apical surface. GPI-linked proteins are able to concentrate in glycosphingolipid-rich domains, and these lipid rafts (also referred to as detergent insoluble glycolipids, or

DIGs), are delivered to the apical surface (Ikonen 2001, Jacobson & Dietrich 1999, Simons & Ikonen 1997). It is thought that apical sorting signals promote partitioning of a protein into apically bound lipid rafts. Support for this model comes from studies of the transmembrane domains (TMD) from the influenza virus hemagglutinin (HA) and neuraminidase (NA) proteins. For both the HA and NA proteins, residues within the TMD play a role in directing apical sorting (Kundu et al. 1996, Lin et al. 1998). An analysis employing chimeric constructs, as well as mutations to the TMD of NA and HA, does not reveal any discrete sequences responsible for mediating raft association and apical sorting. Rather, the results suggest different requirements for raft association and apical sorting and suggest that discontinuous elements, in some cases extending outside the TMD, direct the trafficking of these proteins (Barman et al. 2001). Interactions between the TMD and lipid rafts, or other sorting machinery restricted to lipid rafts, may be required for apical delivery. It is also possible that the sorting information within a TMD interacts with sorting machinery that is independent of lipid rafts.

N-linked glycosylation sites are involved in apical delivery of certain proteins (Benting et al. 1999, Fiedler et al. 1994, Fiedler & Simons 1995, Gut et al. 1998, Scheiffele et al. 1995). It has been proposed that N-linked sugars of the cargo protein may bind with lectin-like proteins exposed in the lumen of the secretory pathway and that the cargo protein is delivered to the apical surface by virtue of sorting information present in the cytoplasmic domain of the lectin-like protein. Recently the N-glycosylation sites present in the large extracellular loop of the neuronal glycine transporter, GLYT2, were shown to mediate apical trafficking in polarized MDCK cells (Martinez-Maza et al. 2001). The mechanism responsible for polarized sorting associated with N-linked glycosylation remains to be clearly established. Regardless of the mechanism, however, N-linked glycosylation is likely to be the primary apical sorting determinant for only a subset of proteins. This is demonstrated by studies of several apical proteins for which N-linked glycosylation clearly does not contribute to proper sorting (Bravo-Zehnder et al. 2000, Marzolo et al. 1997, Meerson et al. 2000, Su et al. 1999).

## **The Activities of Sorting Signals are Hierarchical with Respect to One Another**

It has been suggested that a protein destined for expression at the cell surface, but lacking its sorting determinant, is not sorted at all, and its appearance at both surfaces results from a stochastic partitioning process at the level of the TGN. The absence of sorting information in other cases causes the protein or multisubunit protein complex to fail quality control check points, resulting in its retention and degradation in the ER (Ellgaard & Helenius 2001, Reddy & Corley 1998). However, it often appears that removal of the primary sorting determinant results in the exposure or activation of a previously cryptic sorting determinant. For example, when the basolateral sorting signals of LDL-R and pIg-R are disabled, the proteins are delivered to the apical surface, as opposed to being trapped within the ER or

targeted to both membrane surfaces as would be expected if these proteins possessed only a single sorting determinant (Casanova et al. 1991, Hunziker et al. 1991). Carboxypeptidase M and lactase phlorizin hydrolase provide additional examples of proteins that appear to embody both apical and basolateral sorting information (Jacob et al. 1999, McGwire et al. 1999). The mechanisms that determine the hierarchy according to which multiple signals in a single protein are acted upon remain unelucidated.

## The Cellular Sorting Machinery

The cellular sorting machinery must be able to differentiate between various signals and traffic proteins to the correct surface domains or intracellular compartments. Many of these recognition events are likely to occur at the TGN or in endosomal compartments. A complex of vesicle-coating proteins (COPs) is essential in the anterograde and retrograde movement of vesicles between the ER and Golgi, and between the Golgi cisternae (Barlowe 2000). In addition to COP coats, the adaptor family of proteins is involved in the trafficking of vesicles between the TGN, plasma membrane, and endosomal compartments (Kirchhausen 2002, Robinson & Bonifacino 2001). There are currently four classes of adaptor complexes: AP-1, AP-2, AP-3, and AP-4. The adaptor complexes are tetramers consisting of two large subunits,  $\gamma$  and  $\beta$ , or  $\alpha$  and  $\beta 2$ , an intermediate sized  $\mu$ -subunit, and a small  $\sigma$ -subunit. AP-1 and AP-2 complexes bind to cytoplasmically exposed domains of vesicle cargo proteins and recruit clathrin to the budding vesicles. AP-1 governs transit of vesicles from the TGN to the cell surface, whereas AP-2 is associated with the internalization of vesicles from the cell surface. AP-3 and AP-4 complexes are involved with trafficking to lysosome and endosome compartments (Aguilar et al. 2001; Dell'Angelica et al. 1997, 1999a,b; Obermuller et al. 2002). AP-4 does not appear to recruit clathrin coats, but may instead regulate the formation of non-clathrin coats.

Direct evidence indicating a role for adaptor complexes in protein trafficking came from the finding that  $\mu$ -subunits of adaptor complexes can interact directly with tyrosine-based sorting determinants (Ohno et al. 1995, 1996) and that a di-leucine motif can interact with the  $\beta$ -subunit of the AP-1 adaptor complex (Rapoport et al. 1998). Concurrent studies suggested that tyrosine-based signals were not interpreted identically in all epithelial cell types. A tyrosine-based sorting motif in the  $\beta$ -subunit of the H,K-ATPase is essential for basolateral sorting in MDCK cells. However, this protein is restricted to the apical surface of the LLC-PK<sub>1</sub> epithelial cell line, suggesting that the two cell lines interpret the tyrosine-based sorting motifs differently (Roush et al. 1998). This difference in membrane targeting appeared to be confined to only a subset of basolateral proteins because this work also showed that a di-leucine motif-containing protein, FcR2-B2, was present at the basolateral membrane in both MDCK and LLC-PK<sub>1</sub> cells. Subsequently, a novel AP-1 subunit isoform,  $\mu 1B$ , was identified. The  $\mu 1B$  protein interacts with tyrosine-based determinants and is restricted in its expression to some but not all epithelial and exocrine cells (Ohno et al. 1999). The  $\mu 1B$

subunit is not endogenously expressed in LLC-PK<sub>1</sub> cells. When it is introduced into this cell line, the transfected LLC-PK<sub>1</sub> cells gain the ability to sort proteins containing tyrosine-based sorting signals to the basolateral surface (Folsch et al. 1999). Further work confirmed that the  $\mu$ 1B subunit is sufficient to link cargo proteins bearing basolateral sorting motifs with the AP-1B adaptor complex and clathrin coat proteins (Folsch et al. 2001, Sugimoto et al. 2002). Subsequent studies suggest that  $\mu$ 1B may exert its effects during a protein's post-endocytic sorting rather than during its initial delivery to the plasmalemma (Gan et al. 2002). It will be interesting to determine whether adaptor complexes play a general role in recognizing sorting determinants and in providing the basis for cargo vesicle delivery to specific cell surface domains or organelles.

Once a protein has been packaged into the correct vesicle by the cellular sorting machinery operating at the level of the TGN, the cell next has to determine how to direct that vesicle to the correct surface domain. In the case of the mannose-6-phosphate receptor (M6PR), a plus end-directed microtubule motor protein, KIF13A, interacts with the  $\beta$ 1-adaptin of the AP-1 adaptor complex. As a consequence of this interaction, KIF13A is tethered to M6PR-containing cargo vesicles (Nakagawa et al. 2000). These interactions appear to define a mechanism for delivering the M6PR-containing vesicles budding from the TGN to the plasma membrane along microtubules via the KIF13A motor activity (Nakagawa et al. 2000).

The delivery of cargo vesicles to membrane surface domains, or intracellular compartments, is further regulated by membrane-associated complexes known as SNAREs (Hanson et al. 1997, Hay & Scheller 1997, Nelson & Yeaman 2001, Warren & Malhotra 1998). In short, the SNARE hypothesis posits that cargo vesicles possess vesicular receptor molecules, or v-SNAREs, that match up with target receptors, t-SNAREs, at the appropriate cellular destination. Importantly, some SNARE components, such as the various syntaxin isoforms, have restricted subcellular localizations and therefore could serve as a means to ensure that cargo vesicles dock specifically with the correct cellular target membrane (Brown & Breton 2000, Saxena et al. 2000). In addition to the SNAREs, the Rab GTPases also participate in regulating vesicle trafficking and docking. The literature on these subjects is extensive, and a complete discussion is beyond the scope of this review. There are a number of excellent recent review articles that explore the roles of SNAREs and Rabs in conferring specificity upon vesicular trafficking (Novick & Zerial 1997, Segev 2001, Waters & Pfeffer 1999).

## Stabilization/Retention Post-Surface Delivery

For many membrane proteins, maintenance of the correct localization requires interactions with structural proteins located at or near the plasma membrane. The PDZ domain (acronym derived from the post-synaptic density protein, PSD-95, the *Drosophila* septate junction protein Discs-large, and tight junction ZO-1 protein, all of which contain the characteristic domain) is an 80–90-amino acid protein-protein interaction domain present in many polypeptides (Fanning & Anderson 1999, Hung & Sheng 2002). Proteins containing PDZ domains often interact with

specific sequences within the cytoplasmic tail of integral membrane proteins. The trafficking, stability, and/or activity of a variety of receptors, transporters, and channels are influenced by interactions with PDZ domain proteins (Brown & Breton 2000, Chung et al. 2002, Kim 1997, Sheng & Sala 2001, Voltz et al. 2001). In *Caenorhabditis elegans* the PDZ domain-containing protein, LIN-2, is responsible for the basolateral localization, in vulval precursor cells, of the receptor tyrosine kinase, LET-23 (Simske et al. 1996).

The human homolog of LIN-2, hCASK, is implicated in the basolateral localization of both syndecan-2 and protein 4.1 (Cohen et al. 1998). In both neurons and epithelia, a related PDZ protein, LIN-10, is linked to the correct membrane targeting of the glutamate receptor, GLR-1 (Rongo et al. 1998). The PDZ protein, Lin-7, interacts with a PDZ association motif in the C-terminal cytoplasmic domain of a  $\gamma$ -amino butyric acid (GABA) transporter family member, the betaine transporter, BGT-1 (Perego et al. 1999). This interaction is not required for the targeting of BGT-1 to the basolateral surface of MDCK cells but is required to prevent the re-internalization of the transporter (Perego et al. 1999). These are but a few of the rapidly growing list of proteins whose distributions may be stabilized at specific surface domains in epithelial cells through PDZ interactions. It is likely that PDZ domain-mediated protein-protein interactions play an important role in establishing, maintaining, and regulating the distributions of a wide variety of proteins present at the plasma membrane in both epithelia and neurons.

## Non-Classical Sorting Determinants

In addition to the two most well-characterized basolateral sorting motifs, a number of proteins have been identified that harbor cytoplasmic tail-encoded signals differing from the classic signals. Recently, a novel basolateral sorting signal was characterized in the receptor tyrosine kinase, ERBB2 (Dillon et al. 2002). This C-terminal sorting information consists of a juxtamembrane, bipartite signal located within an 11-amino acid stretch. This region is able to redirect a chimera of the apical neurotrophin receptor to the basolateral surface of MDCK cells. Interestingly, this signal may interact directly with the basolateral-targeting adaptor,  $\mu$ 1B, as the signal does not mediate strong basolateral sorting in LLC-PK<sub>1</sub> cells that lack the  $\mu$ 1B adaptor.

A recent report characterizing the sorting of the apically located megalin receptor in MDCK identifies a stretch of amino acids in the C-terminal cytoplasmic tail that confers apical sorting (Takeda et al. 2003). Surprisingly, the stretch of amino acids that facilitates apical sorting includes two canonical basolateral/endocytosis sorting signals, an NPXY and a YXX $\phi$  motif. Two other NPXY domains in the cytoplasmic C-terminal tail, but outside the region implicated in apical sorting, act as endocytosis signals in megalin. A tripeptide PDZ-interacting domain was not essential for the apical localization of megalin. The exact make-up of the apical determinant has yet to be determined, and whether it overlaps with or requires the tyrosine residues within the basolateral/endocytosis signal is not clear.

## MOLECULAR SIGNALS INVOLVED IN ION TRANSPORT PROTEIN SORTING IN EPITHELIAL CELLS

Not surprisingly, the same signals and mechanisms discussed in the preceding section participate in creating the polarized distributions of at least some epithelial transport proteins. In the case of the renal water channel aquaporin 4 (AQP4), for example, its basolateral distribution is specified by a pair of familiar and well-defined sorting motifs (Madrid et al. 2001). The primary structure of the cytoplasmic C-terminal tail of AQP4 includes a tyrosine-based sequence (YMEV) and a di-leucine-like sequence set in the context of acidic residues (ETEDLIL). Both motifs are capable of functioning as basolateral-sorting signals and both apparently actively participate in establishing the basolateral localization of AQP4. The tyrosine-containing sequence also apparently mediates AQP4 endocytosis and targeting to lysosomes through interactions with AP2 and AP3 adaptor complexes, respectively. It has yet to be determined directly whether the targeting information embedded within the YEVM signal is interpreted through interactions with the  $\mu$ 1B adaptor subunit. However, it seems unlikely that such an interaction is absolutely required to mediate the basolateral sorting of AQP4 because this protein behaves as a basolateral polypeptide when it is expressed in the LLC-PK<sub>1</sub> renal epithelial cell line, which does not produce  $\mu$ 1B (Ohno et al. 1999).

Although the example of AQP4 demonstrates that the classical targeting signals clearly can play a role in transport protein sorting, those transport proteins that employ them appear to be the exception rather than the rule. Perhaps as a consequence of their complex structures or their susceptibilities to manifold regulatory influences, transport proteins appear to rely on a variety of unique signal structures to attain their subcellular distributions.

The inwardly rectifying potassium channel Kir 2.3 spans the membrane twice and is expressed on the basolateral surfaces of renal collecting duct epithelial cells. An 11- amino acid sequence near the C terminus appears to specify vectorial targeting of the channel to the basolateral plasmalemma (Le Maout et al. 2001). The amino acid sequence contains a tyrosine residue, but the tyrosine does not appear in the context of an obvious YXX $\Phi$  or NPXY motif. Although the role of the tyrosine has not been tested directly, it does not participate in direct interactions with the  $\mu$ 1b adaptor subunit. Furthermore, Kir 2.3 behaves as a basolateral protein when expressed in LLC-PK<sub>1</sub> cells, which lack  $\mu$ 1b expression. It is interesting to note that a PDZ-interacting motif at the extreme C terminus of Kir 2.3 is not required for basolateral sorting but does play an important role in maintaining a stable population of the protein at the basolateral surface (Olsen et al. 2002). This behavior is reminiscent of that of the previously discussed betaine transporter, BGT (Perego et al. 1999). Another atypical tyrosine-based basolateral sorting determinant, YKAA, has been identified in the cytoplasmic tail of the hepatic Na<sup>+</sup>/taurocholate cotransporter, Ntcp (Sun et al. 2003). NMR two-dimensional spectroscopy shows that this tyrosine-based motif is unlikely to form a  $\beta$ -turn,

suggesting that this sequence may be interpreted through a non-clathrin adaptor complex sorting pathway.

The Na,K-ATPase, or sodium pump, is far and away the most abundant ion transport protein residing in the basolateral plasma membranes of the majority of epithelial cell types. It is a heterodimer, composed of an  $\alpha$ -subunit that spans the membrane 10 times and a  $\beta$ -subunit that crosses the membrane once in a type II orientation (Dunbar & Caplan 2001). The sodium pump is part of the large P-type collection of ion pumps, whose members include both the ER and plasma membrane Ca-ATPases. Its closest cousin in this family is the gastric H,K-ATPase, the ion pump responsible for acid secretion in the stomach. In stimulated gastric parietal cells, the H,K pump resides in the apical plasmalemma. The distinct sorting patterns of these closely related pumps are recapitulated when they are expressed by transfection in the LLC-PK<sub>1</sub> line of renal epithelial cells. Analysis of the sorting behaviors of chimeras composed of complementary portions of the  $\alpha$ -subunits reveals that pump targeting is determined by a complex and apparently conformation-dependent signal (Dunbar et al. 2000). This signal does not appear to be composed of a single motif. Rather, it is the product of an interaction between the  $\alpha$ -subunit fourth transmembrane domain and the sequences that flank it. The mechanism through which this signal is interpreted remains to be determined. The Na,K-ATPase interacts with elements of the subcortical cytoskeleton through direct associations with ankyrin (Koob et al. 1988, Morrow et al. 1989, Nelson & Veshnock 1987). It has been suggested that this interaction plays a role both in the trafficking of the Na,K-ATPase through the biosynthetic pathway and in its stable incorporation into the epithelial basolateral plasmalemma (Devarajan et al. 1997, Hammerton et al. 1991, Mays et al. 1995). Because multiple separate domains of the  $\alpha$ -subunit appear to contribute to the formation of the ankyrin-binding site (Devarajan et al. 1994), it is possible that the fourth transmembrane segment and its flanking sequences are involved in creating a tertiary structural conformation that favors ankyrin binding. Similarly, a conformation favored by the interaction between the fourth transmembrane segment and its flanking sequences may favor an association between the pump and some other component of the targeting or stabilization machinery.

The signals and mechanisms involved in the sorting of apical transport proteins bear even less apparent relationship to those that have been identified in the studies of the single-pass membrane proteins discussed above. The apical localization of several ion transport systems appears to depend upon interactions between the extreme C termini of these proteins and proteins containing one or more PDZ domains. The apical CFTR chloride channel, for example, interacts with several PDZ domain-containing proteins, including CAL, CAP70, and NHERF (Cheng et al. 2002b; Wang et al. 1998, 2000). The last three residues of the CFTR C terminus constitute the protein's PDZ interaction motif. Deletion of these residues results in basolateral accumulation of the mutant protein. In fact, a similar deletion mutation appears to account for the symptoms of cystic fibrosis (CF) in a small minority of CF patients (Moyer et al. 1999, 2000). The apical Na,Pi cotransporter of the renal

proximal tubular brush border also appears to owe its apical distribution in large measure to PDZ interactions mediated through its C-terminal tail (Hernando et al. 2002, Karim-Jimenez et al. 2001). The CAP70 protein involved in the apical localization of CFTR has been independently identified as an important determinant in establishing the distribution of NaPi cotransporter (Gisler et al. 2001). Finally, the Na- and Cl-dependent GABA transporter, GAT3, behaves as an apical protein when expressed in MDCK cells (Muth et al. 1998). Once again, the extreme C-terminal three amino acids of GAT3 are required for this sorting behavior, and these residues specify an interaction with CAP70. It would appear that, at least in the case of CFTR, the CAP70 interaction does not play a role in biosynthetic sorting because newly synthesized CFTR is delivered randomly from the TGN to both surfaces of the plasma membrane (Swiatecka-Urban et al. 2002). Rather, this association appears to play a critical role in preventing the internalization and degradation of the apical population of CFTR. Thus PDZ interactions may contribute to apical localization primarily by selectively stabilizing transport proteins delivered either randomly or vectorially to the apical membrane.

While clearly important, PDZ interactions are not sufficient to account for the apical localizations of many of proteins from a variety of transporter families. The MRP2 protein, for example, is a member of the multidrug resistance (MDR) transporter family and drives the efflux of anionic organic compounds across the apical membranes of polarized epithelial cells. CFTR is another member of the MDR family, and these two proteins share significant structural homology. Like CFTR, MRP2 possesses a probable PDZ interaction motif TKF at its extreme C-terminus, which appears to embody the information that specifies its apical localization (Nies et al. 2002). However, this information does not reside in the last three amino acids. Deletion of the TKF sequence does not alter the apical localization, rather, a sequence located between 11 and 15 residues upstream of the C terminus seems to play a role in assuring apical distribution of MRP2. The precise molecular definition of the apical localization signal and its mechanism of action have yet to be determined. The existence of another novel apical sorting determinant is suggested by studies of the SGLT1 isoform of the Na<sup>+</sup>-dependent glucose cotransporter. (Suzuki et al. 2001). This apical signal appears to rely upon an aspartic acid residue at position 28 in the transporter's N terminus. Mutation of the residue results in intracellular retention of SGLT1 and is responsible for a subset of cases of a human genetic disease, glucose/galactose malabsorption.

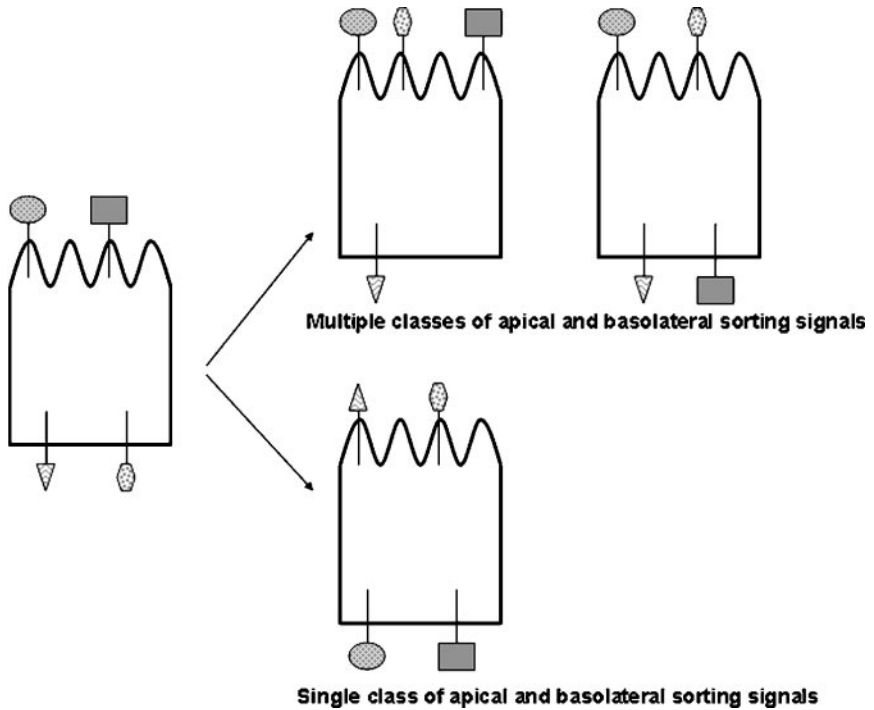
## THE MULTIPLICITY OF SORTING SIGNALS

If it succeeds in no other way, the preceding discussion should at least convey the impression that transport proteins invoke a wide variety of structurally and biochemically diverse motifs to ensure their localization to one of two epithelial plasma membrane surfaces. At first glance, this redundancy seems unnecessarily complicated. After all, epithelial sorting is in essence a binary problem and should

be susceptible to a simple binary solution. Why have epithelial cells gone to the considerable trouble involved in developing the machinery required to interpret and act upon multiple classes of apical or basolateral sorting motifs when each of these classes functions to specify sorting to the same destination? The teleological justification for this rather baroque profusion of sorting signals and pathways may arise from the fact that epithelial cells are called upon to deploy a limited arsenal of transport proteins to achieve a staggering array of transport functions. The V-type H-ATPase, for example, drives acid secretion into the urine when it is inserted into the apical plasma membranes of the  $\alpha$ -type intercalated cells of the renal collecting tubule (Al-Awqati 1996). This same pump catalyzes base secretion into the urine when it is a component of the basolateral plasma membranes of the collecting tubule's  $\beta$ -type intercalated cells. The sodium pump is also subject to differential distribution. In most epithelial cells it occupies the basolateral membrane; however, in the choroid plexus and other epithelia derived from neuroectoderm, the Na,K-ATPase behaves as an apical protein (Rizzolo 1999). This plasticity demands the existence of multiple signals. If sorting were specified by only a single pair of complementary signals, then an epithelial cell could not accomplish the redistribution of one particular basolateral protein to the apical surface without simultaneously effecting this redistribution for its entire complement of basolateral proteins (see Figure 2). The capacity of any polarized epithelial cell type to mediate a specific transport process is predicated upon its capacity to deliver the appropriate transport proteins to its apical and basolateral surfaces. The same transport proteins may be called upon to serve as apical or basolateral proteins in order to fulfill the transport missions assigned to each of the many different epithelial cell types. Thus it is probably inaccurate to associate membrane protein sorting signals with specific destinations. Rather, these signals identify the proteins that display them as members of a class, each of whose members are sorted in concert. The actual destination to which a given class is directed is chosen by the epithelial cell and is determined at the molecular level by the components of the sorting machinery that the epithelial cell expresses. In this manner, each type of transporting epithelial cell can differentially distribute transport proteins so as to achieve the localizations required by its particular physiologic role. This flexibility is a hallmark of transport epithelia structure and function, and it may well owe its existence to the seeming over-abundance of sorting signals.

## MECHANISMS AND SIGNALS IN NEURONAL POLARITY

Directing channels, receptors, transporters, and other synaptic elements correctly to either axonal or somatodendritic domains is an essential prerequisite for proper neuronal function. The similar requirements for biochemical polarity in both neurons and epithelia led investigators to test the possibility that sorting signals and sorting mechanisms are shared between the two cell types (Dotti & Simons 1990). Early evidence suggested that when epithelial proteins were expressed in neuronal



**Figure 2** A multiplicity of sorting signals permits epithelial cells to adapt their protein-sorting properties to their physiologic tasks. If all epithelial cells had available to them only a single type of signal to specify sorting to the apical or basolateral domain, it would not be possible for diverse epithelial cell types to differentially distribute individual components of their membrane protein complements. If a cell's role required that one normally apical protein be directed to the basolateral plasmalemma, then the cell's entire inventory of apical proteins would need to be similarly redirected (*bottom panel*). In contrast, if cells can employ multiple classes of signals to specify sorting, then individual collections of membrane proteins can be redirected without influencing the distributions of unrelated polypeptides (*top panel*).

cells, targeting to the axonal process of neurons was related to the targeting to the apical surface of epithelia. Furthermore, targeting to the somatodendritic surface of neurons was related to targeting to the basolateral surface in epithelia. The initial sets of experiments comparing sorting in epithelia and neurons used single-pass membrane proteins as markers for axonal and dendritic sorting. In order to extend these studies to polytopic membrane proteins and to focus on proteins of significant physiologic relevance to neurons, several studies examined the sorting of isoforms of the GABA transporters (GATs) (Ahn et al. 1996, Gu et al. 1996, Pietrini et al. 1994). In polarized rat hippocampal neurons grown in culture, the GAT-1 protein

is restricted to the axonal membrane. When this transporter is expressed in the polarized epithelial MDCK cells, the transporter is sorted to the apical surface (Pietrini et al. 1994). Analysis of other GATs showed that the BGT-1 transporter, an endogenous basolateral membrane protein from MDCK, is expressed predominantly in somatodendritic membranes of cultured hippocampal neurons following microinjection of its cDNA (Ahn et al. 1996). Similarly, a sorting domain in the C-terminal cytoplasmic tail of the serotonin receptor, 5-HT(1B)R, directs axonal sorting in hippocampal cells and apical sorting in LLC-PK<sub>1</sub> epithelia (Gu et al. 1996, Jolimay et al. 2000); two di-leucine motifs in the C-terminal tail of the glycine transporter GLYT1b direct basolateral sorting in MDCK and somatodendritic sorting in hippocampal neurons (Zafra & Gimenez 2001).

However, there are also several examples of proteins that do not fit the paradigm associating apical with axonal and basolateral with somatodendritic sorting. The metabotropic glutamate receptor, subtype 7a, carries a sorting domain in its cytoplasmic tail that is required for basolateral sorting in MDCK cells. When appended to a surface marker protein in hippocampal neurons, however, it is expressed uniformly in axonal and somatodendritic regions (McCarthy et al. 2001). Work with the excitatory amino acid transporter 3 (glutamate transporter EAAT3) identifies a C-terminal region sufficient for apical sorting in MDCK (Cheng et al. 2002a). In studies of EAAT chimeras this C-terminal region of EAAT3 was able to direct the normally basolateral EAAT1 and the nonpolarized EAAT2 to the apical surface. Surprisingly, this same C-terminal region is necessary for the somatodendritic localization of EAAT3 in hippocampal neurons (Cheng et al. 2002a). An N-myristoylation modification to the post-synaptic density protein, PSD-Zip70, is necessary for dendritic synapse targeting, but it also mediates apical targeting in MDCK epithelia (Konno et al. 2002). Other isoforms of the glycine transporters also exhibit sorting patterns that do not fit the apical/axonal and basolateral/somatodendritic paradigm in epithelia and neurons (Poyatos et al. 2000). It is clear that despite the sorting similarities between neurons and epithelia, apical and axonal sorting determinants and basolateral and somatodendritic determinants are not always interchangeable.

## MEMBRANE TRAFFICKING IN THE REGULATION OF ION TRANSPORT PROTEIN FUNCTION

Recently, it became clear that the processes that orchestrate the polarized distributions of transport proteins may also regulate their functions. Several transport proteins are not constitutive components of a particular cell surface domain. Instead, these proteins commute between the plasmalemma and an intracellular storage compartment. In response to changes in intracellular second messenger concentrations, transporters are either inserted into or retrieved from the plasmalemma. By manipulating the surface populations of selected transport proteins, epithelial cells can precisely modulate their physiologic properties. The signals and pathways

responsible for this regulated trafficking may be closely related to those that determine polarized localizations of these transport proteins.

As noted above, acid secretion in the stomach is driven by the gastric H,K-ATPase. In the resting state, the majority of H,K-ATPase is restricted to the tubulovesicular elements (TVEs) of the gastric parietal cells. This extensive system of membranous vesicles resides beneath the plasma membrane and serves as an intracellular storage compartment that is densely packed with quiescent H,K pumps. Upon stimulation of the parietal cell by secretagogues, there is a transient rise in the intracellular levels of cAMP, IP<sub>3</sub>, diacylglycerol, and Ca<sup>2+</sup>. Some combination of these second messengers induces the TVEs to fuse with the apical plasma membrane, thus allowing the H,K-ATPase to secrete protons directly into the lumen of the gastric gland (Hersey & Sachs 1995). Upon withdrawal of stimulation, the enzyme, along with large portions of the plasma membrane, is re-internalized to create the TVEs. The  $\beta$ -subunit of the gastric H,K-ATPase contains a tyrosine-based endocytosis motif that is necessary for the re-internalization of the holoenzyme (Courtois-Coutry et al. 1997). Transgenic mice expressing H,K-ATPase  $\beta$ -subunit in which the critical tyrosine residue is mutated to an alanine fail to re-internalize the enzyme, leading to hyper-secretion of acid and chronic gastritis. Conversely, the parietal cells of mice that carry a disruption of the H,K-ATPase  $\beta$ -subunit gene are completely devoid of TVEs, suggesting that the TVE compartment is generated or maintained through endocytotic events that are, in turn, dependent upon the H,K-ATPase  $\beta$ -subunit.

Insulin acts upon skeletal and cardiac muscle cells and adipocytes to induce a dramatic increase in the glucose permeability of the sarcolemma. This effect is accomplished through the insertion into the plasma membrane of vesicles whose membranes contain the GLUT4 glucose transport protein (Bryant et al. 2002). This membrane fusion process involves components of the SNARE machinery and is modulated by Munc18, which appears to regulate the assembly and fusion competence of SNARE complexes (Khan et al. 2001, Thurmond & Pessin 2000). Furthermore, translocation of Glut4-containing vesicles to the plasmalemma appears to involve the activity of the unconventional myosin motor protein MyoIc (Bose et al. 2002). Retrieval of GLUT4 from the cell surface upon the withdrawal of insulin stimulation appears to depend upon endocytosis motifs that are present in both the N and C termini of the GLUT4 polypeptide (Shewan et al. 2000).

A number of renal transport proteins subject to regulation by mechanisms involving membrane trafficking has also been documented. Among the most thoroughly investigated are the vacuolar H-ATPase, the aquaporin 2 water channel (AQP2), and the sodium phosphate cotransporter, NaPiII (Brown 2000). These three proteins represent distinct classes of transport systems that are expressed in different renal cell types and are induced to participate in regulated membrane trafficking events through diverse signaling pathways. Taken together, their behaviors provide a useful illustration of the manifold renal adaptations that exploit membrane trafficking processes as tools with which to effect acute physiologic responses.

The vacuolar H-ATPase expressed by the intercalated cells of the renal collecting tubule plays a critical role in maintaining systemic acid-base balance (Al-Awqati 1996). This protein complex is composed of a large number of subunit polypeptides, several of which assemble into a transmembrane proton permeation pathway, whereas the remainder interact at the cytoplasmic aspect of this pore to form an ATP-driven molecular motor that powers proton translocation (Nishi & Forgac 2002). The vacuolar H-ATPase mediates the acidification of intracellular organelles, including endosomes and lysosomes, and it pumps acid across the plasma membranes of intercalated and proximal tubule epithelial cells. Inactivating mutations in genes encoding isoforms of proton pump subunit polypeptides account for a subset of cases of human familial renal tubular acidosis (Karet et al. 1999).

The subcellular distribution of the H-ATPase in intercalated cells is complex and variable. As noted above, in acid-secreting  $\alpha$ -type intercalated cells, the H-ATPase is found at the apical plasma membrane, as well as in a population of intracellular vesicles (Al-Awqati 1996). In contrast, in bicarbonate-secreting  $\beta$ -type intercalated cells, the H-ATPase is found in intracellular vesicles and at the basolateral plasma membrane. The intracellular population of H-ATPase can be mobilized to the apical or basolateral cell surfaces in response to acidosis or alkalosis, respectively. This adaptive redistribution appears to involve fusion of the intracellular H-ATPase-containing storage vesicles with the plasmalemma. Cultured inner medullary collecting duct cells appear to be able to recapitulate the redistribution of the intracellular H-ATPase to the apical plasma membrane *in vitro*. Exposing these cells to an environment that causes cytoplasmic acidification leads to a net increase in the quantity of H-ATPase activity present in the plasma membrane. Furthermore, this increase is inhibitable by preincubation of the cells with clostridial toxins, suggesting that it is the product of SNARE-mediated membrane fusion events (Alexander et al. 1997). Endocytic re-internalization of the proton pump is thought to accompany the down-regulation of proton and bicarbonate transport that follows recovery from the acidotic or alkalotic state. The mechanisms through which intercalated cells sense acid-base status and the cellular machinery involved in orchestrating the membrane trafficking events associated with their response remain largely unknown.

AQP2 is expressed in the principal cells of the renal collecting tubule and, like the other members of the large aquaporin family, it forms a homotetrameric water channel (Deen et al. 2000). In the absence of antidiuretic hormone (ADH), AQP2 resides in the membranes of intracellular vesicles. Hormonal stimulation either increases the rate of fusion of these vesicles with the apical plasma membrane or decreases the rate of endocytosis of the AQP2 protein, resulting in a net increase in the number of AQP2 channels present in the membrane domain. This, in turn, increases the *trans*-epithelial hydraulic conductivity and permits water re-absorption, driven by the medullary osmotic gradient, to proceed. Removal of the hormone results in net re-internalization of the apical AQP2 population and with it a concomitant reduction in the capacity to create a concentrated urine.

Binding of ADH to its receptor leads to elevations of cAMP, which activate protein kinase A, leading to phosphorylation of a serine residue present in the AQP2 N-terminal cytoplasmic tail. Activation of vesicular delivery to the cell surface requires that multiple monomers in the AQP2 homotetramer become phosphorylated (Kamsteeg et al. 2000). Although AQP2 vesicles clearly contain elements of the SNARE membrane fusion machinery (Inoue et al. 1998), the mechanism whereby multiple phosphorylation of AQP2 tetramers is coupled to vesicular translocation remains unelucidated.

The sodium-coupled phosphate transporter NaPiIIa is a denizen of the apical brush borders of renal proximal tubule epithelial cells, where it serves as the kidney's principal pathway for phosphate re-absorption (Murer et al. 2003). One of the physiologic effects of parathyroid hormone (PTH) is to increase renal phosphate clearance. Activation of the PTH receptor leads to the rapid endocytosis of NaPi, thus diminishing the proximal tubule's phosphate transport capacity. Following this internalization, NaPiIIa is targeted to lysosomes for degradation. In contrast to the H-ATPase and AQP2, which undergo multiple rounds of internalization, intracellular vesicular storage, and regulated plasma membrane insertion, the influence of PTH on NaPiIIa trafficking appears to be irreversible. While the cellular machinery responsible for the PTH-induced endocytosis of NaPiIIa has yet to be identified, interesting recent studies have identified a di-basic motif present in one of the cytoplasmic loops that appears to play a critical role in specifying the response to PTH induction (Karim-Jimenez et al. 2000). Although di-basic motifs have not previously been shown to be involved in endocytic signaling, this sequence is both necessary to ensure the PTH-responsive internalization of NaPiIIa and sufficient to confer this property upon NaPiIIb, an isoform of the sodium phosphate cotransporter that does not normally undergo a PTH-driven translocation.

In addition to the three well-known examples discussed above, there is a long and growing list of renal transport proteins that have been proposed as candidates for regulation through endo- and exocytosis. This list, which includes the Na,K-ATPase and the NHE3 isoform of the sodium-proton exchanger, has accumulated as investigators have sought to elucidate the cellular and molecular processes that account for the kidney's responses to a wide variety of physiologic stimuli. Compelling data have been presented suggesting that both of these ion transport proteins respond to physiologically relevant stimulus by undergoing alterations in their subcellular distributions in proximal tubule epithelial cells.

The Na,K-ATPase is a heterodimeric enzyme composed of a catalytic  $\alpha$ -subunit that spans the membrane 10 times and a glycosylated  $\beta$ -subunit that spans the membrane once (Caplan 1997a). The activity of the Na,K-ATPase in the renal proximal tubule is reduced in response to dopamine (Bertorello & Aperia 1990). Dopamine is synthesized within the proximal tubule epithelial cells themselves and is released into the tubule fluid (Aperia 2000). Interaction with the DA1 dopamine receptor present in the apical membranes of the proximal tubule cells activates a

signaling cascade that leads ultimately to a decrease in sodium re-absorption and a substantial diuresis. The dopamine-induced inhibition of sodium pump activity accounts for a significant portion of this effect. The mechanism through which dopamine exerts its influence over sodium pump function remains the subject of intense research.

Dopamine-induced inhibition of sodium pump activity appears to involve the action protein kinases (Efendiev et al. 1999, Li et al. 1998). The Na,K-ATPase rat  $\alpha$ -subunit is clearly a substrate for phosphorylation by protein kinase C (PKC) (Bertorello et al. 1991, Therien & Blostein 2000). A large body of evidence indicates that this phosphorylation is intimately involved in renal sodium pump response to dopamine. Cell fractionation studies performed on the OK line of opossum kidney proximal tubule epithelial cells suggest that exposure to dopamine causes these cells to internalize as much as 50% of their surface population of Na,K-ATPase (Chibalin et al. 1997). Rat Na,K-ATPase is also subject to this endocytosis when exogenously expressed by transfection in OK cells (Chibalin et al. 1998a). Mutation of the PKC site in the rat  $\alpha$ -subunit renders it insensitive to the down-regulation and apparent internalization induced by dopamine (Chibalin et al. 1999). It has since been shown that the endocytosis of Na,K-ATPase from the basolateral plasmalemma appears to require the involvement of class I(A) phosphoinositide-3 kinase (PI3K) (Chibalin et al. 1998b). PKC phosphorylation of the Na,K-ATPase  $\alpha$ -subunit apparently permits PI3K to bind to an adjacent  $\alpha$ -subunit proline-rich sequence via a SH3 domain in the p85 subunit of PI3K (Yudowski et al. 2000). This binding event may then recruit clathrin adaptor complexes (Ogimoto et al. 2000), leading to the internalization of the Na,K-ATPase and the down-regulation of transport function.

The effects of PTH on the proximal tubule are not limited to its negative influence on Na-coupled phosphate uptake. PTH also substantially reduces  $\text{NaHCO}_3$  re-absorption by decreasing the activity of the apical NHE3 sodium-proton exchanger (Collazo et al. 2000, Zhang et al. 1999). The mechanism of this inhibition appears to be complex and multiphasic. The fast component of the PTH effect appears to involve direct phosphorylation of NHE3 and consequent inhibition of its transport activity (Fan et al. 1999). Recently, evidence from studies performed in OK cells suggests that the later phase is attributable to internalization of NHE3 from the apical plasmalemma (Collazo et al. 2000). Cell surface biotinylation experiments demonstrate that the quantity of surface-exposed NHE3 decreases substantially after 30 min of PTH treatment. Furthermore, this disappearance does not occur in cells transfected with a dominant-negative mutant form of dynamin that inhibits endocytosis. Endocytic down-regulation of both NHE3 and NaPiIIa is also impaired in a mouse model of Dent's disease (Piwon et al. 2000). Dent's disease is caused by mutations that disable the CIC5 chloride channel, which is found in the membranes of subapical endosomes in the renal proximal tubule (Gunther et al. 1998). This channel appears to mediate the chloride flux that is required to permit these endosomes to acidify. In its absence, both acidification and endocytosis are blocked.

## BIOSYNTHETIC ASSEMBLY OF ION TRANSPORT PROTEINS AND DEPARTURE FROM THE ER

Functional channels, receptors, or pumps are often multimeric complexes assembled from multiple subunits. Often multiple identical, or nearly identical, subunits come together to form an active complex. In many cases, subunit assembly takes place within the ER, and improperly assembled complexes are held there until they can be rearranged, or are diverted to degradation pathways (Chevet et al. 2001, Ellgaard & Helenius 2001). Recently there has been great progress made in determining how this assembly is coordinated, and several trafficking elements have been identified that regulate proteins on their route from the ER to the cell surface.

ATP-sensitive potassium channels ( $K_{ATP}$  channels) function as octamers formed from four inwardly rectifying  $K^+$  subunits and four sulfonylurea receptor subunits that regulate channel activity (Babenko et al. 1998). There are two isoforms of the  $K^+$  channel subunit (Kir6.1 and Kir6.2) and three isoforms of SUR (SUR1, SUR2A, and SUR2B). Although heteromultimers containing both Kir6.1 and Kir6.2 isoforms are functional, there are restrictions to the combinations of SUR subunits that result in functional channels (Giblin et al. 2002, Pountney et al. 2001). In order to ensure that only functional channels reach the surface, these channels possess ER retention or retrieval signals that prevent improperly or incompletely assembled complexes from exiting the ER (Ma & Jan 2002). The retention motif, RKR, is present in regions of Kir6 and SUR, which are exposed to the cytoplasm, and it is believed that the RKR signal becomes inaccessible following the assembly of an octameric channel. Appending the RKR sequence to CD4, a protein normally routed to the cell surface, leads to accumulation of the fusion construct within the ER (Zerangue et al. 1999). This ER retention motif differs in sequence from previously identified ER retrieval sequences, KDEL or KKXX, and its activity does not appear to be dependent on its placement within the C-terminal tail of a protein (Nilsson & Warren 1994).

Mutations associated with persistent hyperinsulinemic hypoglycemia recently have been linked to improper assembly of Kir6 and SUR subunits (Cartier et al. 2003, Conti et al. 2002, Taschenberger et al. 2002). In two cases, mutations in the SUR subunit that resulted in trapping of Kir6 and SUR in the ER could be overcome by eliminating the arginine-based ER retention motifs (Conti et al. 2002, Taschenberger et al. 2002). Interestingly, the  $\Delta F508$  mutation of the CFTR chloride channel, which is functional but misfolded and is trapped in the ER, could be directed to the cell surface by deleting four arginine-based ER retention signals (Chang et al. 1999). ER retention signals also play a role in signaling the state of folding or assembly in voltage-gated  $Ca^{2+}$  channels and NMDA and GABA<sub>B</sub> receptors (Bichet et al. 2000, Calver et al. 2001, Cornet et al. 2002, Margeta-Mitrovic et al. 2000, Perez-Otano et al. 2001, Scott et al. 2001, Standley et al. 2000, Xia et al. 2001).

Another class of trafficking determinants promotes the export of proteins from the ER (Nishimura & Balch 1997, Sevier et al. 2000). Recently, related ER export

signals, defined by the FCYENE sequence, have been described in Kir1.1, Kir2.1, Kir3.2A, and Kir3.4 (Ma et al. 2001, 2002; Stockklausner et al. 2001). The Kir3.1 subunit does not have its own ER export signal, and therefore, when expressed alone, it does not reach the cell surface. However, if Kir3.1 is expressed along with Kir3.2A or Kir3.4, the heterotetrameric channels are able to exit the ER because of the ER export motif present in Kir3.2A or Kir3.4 (Ma et al. 2002). Domains containing ER export information have also been identified in SUR1 and in the inwardly rectifying potassium channel Kv1.4 (Li et al. 2000, Sharma et al. 1999). The potassium channel, KCNK, contains a di-basic sequence in its N terminus that interacts with  $\beta$ -COP and results in the channel being retrieved to the ER (O'Kelly et al. 2002). Residues in the C terminus of the KCNK interact with an isoform of the cytoplasmic protein 14-3-3. This interaction is necessary to override ER retrieval mediated by the N-terminal domain and permits the channel to move out of the ER (O'Kelly et al. 2002). Whether the activity of these two opposing signals is regulated in a temporal or hierarchical manner is not completely clear.

## DISEASES AND DYSFUNCTION OF TRANSPORT PROTEIN TRAFFICKING

In light of both their inherent physiologic significance and the complexity of the cellular mechanisms that regulate their distributions and functions, it should come as no surprise that a number of human genetic diseases are attributable to perturbations in the sub-cellular trafficking of transport proteins. In many cases, elucidating the molecular basis of these conditions has illuminated completely new and unanticipated signals and pathways. In a few instances, these insights have suggested new therapeutic strategies aimed at rectifying an underlying targeting defect and thus ameliorating at least some of the symptoms that arise from its dysfunction.

The epithelial sodium channel (ENaC) is expressed in the apical plasma membranes of the principal cells of the renal collecting tubule. The channel is a multimer, composed of some combination of three subunits,  $\alpha$ ,  $\beta$ , and  $\gamma$ , each of which spans the membrane twice. It is responsible for the re-absorption of the final  $\sim 10\%$  of the filtered sodium load, and its activity also provides the electrical driving force for net potassium secretion (Alvarez de la Rosa et al. 2000). Consequently, ENaC is subject to tight regulation by several hormones, including aldosterone. Patients with Liddle's syndrome, an autosomal dominant disorder, exhibit profound hypertension caused by renal sodium retention and the consequent expansion of body fluid volume (Rossier et al. 2002). Although the same complex of symptoms could arise from inappropriately high levels of aldosterone production, patients with Liddle's syndrome exhibit very low serum aldosterone concentrations, leading to the designation of this condition as pseudohyperaldosteronism. Genetic studies have revealed that Liddle's syndrome is caused by mutations that truncate or alter the sequence of the C termini of either the  $\beta$  or  $\gamma$  ENaC subunits (Hansson et al.

1995). It has since been shown that disease-causing mutations disrupt a novel motif containing a tyrosine and several proline residues that is responsible for the internalization and down-regulation of the ENaC channel. Perturbation of this signal produces a channel that is constitutively active at the apical membrane, resulting in excessive sodium retention. Yeast two-hybrid screening revealed that Nedd-4, a component of the ubiquitin ligase machinery, interacts with the tyrosine/proline motif through a WW domain (Staub et al. 1996). Experiments performed in *Xenopus* oocytes suggest that SGK, an aldosterone-induced kinase, may regulate ENaC function by phosphorylating Nedd-4 and thus preventing its interaction with the channel (Debonneville et al. 2001). It remains to be determined whether ENaC down-regulation in epithelial cells in situ is similarly dependent upon Nedd-4 and ubiquitin or is instead orchestrated through more conventional endocytic pathways (Alvarez de la Rosa & Canessa 2003, Shimkets et al. 1997).

A variety of mutations can give rise to the perturbed trafficking of the AQP2 water channel that accounts for the symptoms of nephrogenic diabetes insipidus (NDI) (Deen et al. 2000). Under normal circumstances, antidiuretic hormone (ADH) acts through its receptor to induce the translocation of AQP2 from an intracellular vesicular storage compartment to the apical membranes of renal principal cells, increasing their hydraulic conductivity and permitting osmotic water re-absorption to proceed. Mutations in the genes encoding either the ADH receptor or AQP2 can result in the production of proteins that are retained in the ER and thus not capable of performing their physiologic functions. In either case, the water permeability of the apical membranes of the collecting tubule epithelial cells can not be increased, and large volumes of water are lost in the urine.

Perhaps the best known and most devastating disease-causing mutations that alter the trafficking of a transport protein are those associated with CF. Although CF patients can carry any of hundreds of different mutations in their CFTR genes, the most prevalent by far results in the deletion of a phenylalanine residue at position 508 ( $\Delta F508$ ) (Rosenstein & Zeitlin 1998, Skach 2000). Almost 90% of CF patients carry at least one  $\Delta F508$  CFTR allele. The  $\Delta F508$  mutation results in the production of a misfolded CFTR protein, which is retained in the ER through interactions with the chaperone proteins that constitute the ER quality control machinery (Kopito 1999). Although  $\Delta F508$  CFTR is able to function as a cAMP-gated chloride channel, it does not reach the apical plasma membranes of airway, pancreatic, and intestinal epithelial cells. Consequently, salt and water secretion by these epithelia is compromised, resulting in the production of the thickened mucous that leads to many of the chronic manifestations characteristic of CF.

A number of efforts are under way to develop small molecule pharmacotherapies that will either help the mutant protein to acquire its appropriate tertiary structure or interfere with the ability of the ER chaperones to recognize and detain the  $\Delta F508$  polypeptide. The ER functions as the major repository of mobilizable calcium stores (Meldolesi & Pozzan 1998). Consequently, the concentration of free calcium in the lumen of the ER may be in the millimolar range. Many of the

ER chaperones are calcium-binding proteins (Nigam et al. 1994) and, at least in some cases, high concentrations of calcium are required to permit them to function optimally (Trombetta & Parodi 1992). The ER lumen's calcium stores are generated and maintained by calcium pumps that belong to the Na,K-ATPase family. These sarcoplasmic reticulum/ER calcium pumps (SERCA) can be inhibited by a number of compounds, resulting in the depletion of the ER calcium store.

Some of SERCA pump inhibitors have been tested for their ability to release  $\Delta F508$  CFTR from the ER in human CF cells. Several different structurally diverse Ca-ATPase inhibitors are able to release a cohort of  $\Delta F508$  CFTR to the surfaces of a variety of different human CF cell lines (Egan et al. 2002). The released  $\Delta F508$  CFTR protein functions as a chloride channel and dramatically increases the chloride permeability of the plasma membranes of these CF cells. Remarkably, these compounds are well tolerated and therapeutically beneficial in animal models of CF. A genetically modified mouse that is homozygous for the  $\Delta F508$  mutation exhibits a markedly abnormal electrical potential across its nasal epithelium. Treatment with inhaled calcium pump inhibitors almost completely normalizes this parameter. These data suggest that compounds capable of blocking calcium transport into the ER may alter the environment prevailing in the lumen of the ER in a manner sufficient to frustrate the chaperone proteins and thus permit the escape of some fraction of newly synthesized  $\Delta F508$  CFTR.

Whether this approach to the treatment of CF ever proves useful in a clinical setting, it still serves as a satisfying illustration of the theme first stated in the introduction to this review. The word transport can have multiple meanings to cell biologists and physiologists, but these distinct definitions cross each other's paths in a variety of surprising ways. In this case, inhibition of the ion transport catalyzed by the SERCA Ca-ATPase appears to permit the transport of  $\Delta F508$  CFTR protein to the cell surface, where it can mediate the transepithelial transport of chloride ions that drives fluid and electrolyte secretion in a variety of tissues. Cell biologic trafficking pathways are called upon to regulate the activity of many, if not most, ion transport proteins. The activities of numerous ion transport proteins are critically involved in manifold membrane trafficking processes. It is legitimate to expect that future research into the signals and mechanisms that regulate the distributions and functions of ion transport proteins will continue to expand the overlap between these two definitions and will draw the fields that lay claim to them ever closer together.

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