Chapter 7
Molecular Mechanisms of Apical and Basolateral Sorting in Polarized Epithelial Cells

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Abstract The proper localization of ion channels and transporters to the apical or basolateral membrane domains is essential for correct ion transport through epithelia. Polarized sorting is achieved through elaborate intracellular sorting pathways encompassing the *trans*-Golgi network, early endosomes, and recycling endosomes that are fine-tuned for epithelial function and thus different from the secretory and endocytic systems in non-polarized cells. Polarized cells express epithelial cell-specific molecules, including sorting adaptors like the cytosolic clathrin adaptor AP-1B, that are required for efficient segregation and targeting of proteins to specific plasma membrane domains. In this book chapter, we will first discuss the various sorting stations in the cells followed by a review of apical and basolateral sorting signals and their interpretation at different compartments. Specific emphasis is placed on the trafficking of ion transporters.

Keywords Membrane traffic • Kidney • Biosynthetic • Endocytosis • Recycling • *Trans*-Golgi network • Endosome • AP-1B • Apical • Basolateral

Abbreviations

AEE	Apical early endosome
ARE	Apical RE
BEE	Basolateral early endosome
CRE	Common RE
RE	Recycling endosome

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TGN *trans*-Golgi network
TJ Tight junction

7.1 General Organization of Secretory and Endocytic Pathways

Typically, transmembrane proteins begin their journey to the cell surface in the endoplasmic reticulum (ER) during co-translational insertion into the ER membrane. In this compartment, N-glycans are added to consensus sequences within the protein ectodomains, and proteins are folded and in many cases assembled into oligomers. Properly folded proteins are transported via a coat protein complex II (COPII)-mediated pathway to the Golgi apparatus where they achieve their final glycosylation patterns. The most distal compartment of the Golgi complex, the *trans*-Golgi network (TGN), is the first major sorting station in the cells. From there, proteins may traffic to lysosomes, endosomes, and the cell surface. On their journey to the cell surface, they may traverse early or recycling endosomal populations. Often, the same sorting signals that are used during biosynthetic delivery are also used during endocytic recycling of transmembrane proteins (Matter et al. 1993; Potter et al. 2006b). In the case of polarized cells, an additional level of sorting exists to selectively direct proteins to and from the apical (luminal) and basolateral plasma membranes [reviewed in (Ang and Fölsch 2012; Fölsch et al. 2009; Rodriguez-Boulan et al. 2005)].

Much of the work on polarized sorting in epithelial cells has been performed in the Madin-Darby canine kidney (MDCK) cell line, derived in the 1950s from a female cocker spaniel. The type II subclone of these cells is easily and rapidly cultured as well-differentiated monolayers when plated on permeable support membranes. Moreover, these cells express highly regular microvilli similar to that observed in the brush border of the proximal tubule and elaborate a primary cilium. In MDCK and other cells of kidney origin, most newly synthesized apical and basolateral proteins are targeted vectorially (albeit not necessarily directly) to the plasma membrane. The fidelity of delivery to a given domain is never 100 %, and transcytotic retrieval pathways exist to reroute missorted proteins (Casanova et al. 1991). Other epithelial cell types make more use of this transcytotic pathway to deliver newly synthesized apical proteins. In hepatocytes, almost all transmembrane proteins are delivered first to the sinusoidal (basolateral) membrane, and bile canalicular (apical) targeting follows after internalization (Bartles et al. 1987). An exception to this rule is the class of polytopic membrane proteins, including the bile acid transporters, which are targeted vectorially from the TGN to the apical surface of liver cells (Sai et al. 1999). Intestinal cells, including the polarized Caco-2 cell line, also use the transcytotic pathway to a significant extent to deliver apical proteins, but a sizable fraction of some proteins is also targeted via vectorial pathways (Le Bivic et al. 1990; Matter et al. 1990). In the remainder of this book chapter, we will focus our attention on kidney cells and particularly on knowledge gained using MDCK cells.

After initial delivery to the apical or basolateral plasma membrane, transmembrane receptors may be internalized into early endosomes underlying the plasma membranes. Notably, epithelial cells maintain apical early endosomes (AEEs) and basolateral early endosomes (BEEs) that are biochemically distinct and physically segregated. Indeed, AEEs and BEEs did not fuse with one another in an in vitro fusion assay (Bomsel et al. 1990). Moreover, after treatment of MDCK cells with latrunculin B to disrupt the actin cytoskeleton, AEEs and BEEs were misplaced and intermixed in the cytoplasm, without undergoing fusion reactions (Sheff et al. 2002). Although it is currently unclear which proteins define AEEs and BEEs, their existence probably ensures correct delivery of internalized receptors back to their membrane of origin during rapid recycling from AEEs or BEEs.

In addition to rapid recycling, transmembrane receptors may be sorted further into the cells to reach lysosomes in case they are destined for degradation or recycling endosomes (REs) for sorting back to either their membrane of origin or the opposing membrane during apical-to-basolateral or basolateral-to-apical transcytosis. Unlike in non-polarized cells, REs in polarized cells constitute a second major sorting station of equal importance to the TGN. Indeed, REs in polarized cells are organized into subdomains to accommodate the various sorting needs of cargo in polarized epithelial cells. Two views of REs in MDCK cells have emerged based on conflicting data. In one model, REs are considered to be a single population of interconnected endosomal membranes with different subdomains specialized for targeting to diverse compartments including domains that cluster cargo destined for the apical or basolateral domains [Fig. 7.1b, reviewed in (Ang and Fölsch 2012)]. Notably, the basolateral targeting domain depends on and contains proteins that interact with the AP-1B clathrin adaptor (Fig. 7.1d; see also Sect. 7.3.4). The second model holds that two classes of REs exist: a transferrin receptor (TfnR)-positive common RE (CRE) that can receive internalized cargo from both apical and basolateral surface domains and a specialized apical RE (ARE), marked by Rab11a, that excludes basolaterally recycling cargo and communicates with the CRE and the apical domain [Fig. 7.1a, reviewed in (Mostov et al. 2003)]. These models may not be mutually exclusive, and it is possible that the structure of the RE system in MDCK cells reflects their differentiated state and the need for communication between apical and basolateral surface domains. From here on, we will use the terms ARE and CRE or simply just REs interchangeably. Furthermore, if not noted otherwise, reviewed studies were performed in MDCK cells.

7.2 Sorting to the Apical Membrane

A wide variety of transporters, enzymes, and receptors are present at the apical surface of kidney cells. The steady-state distribution of these proteins can be modulated by changes in the fidelity of biochemical and post-endocytic sorting as well as by alterations in the kinetics of delivery and removal from the surface. Dissecting the sorting signals on proteins that guide them to the apical or basolateral

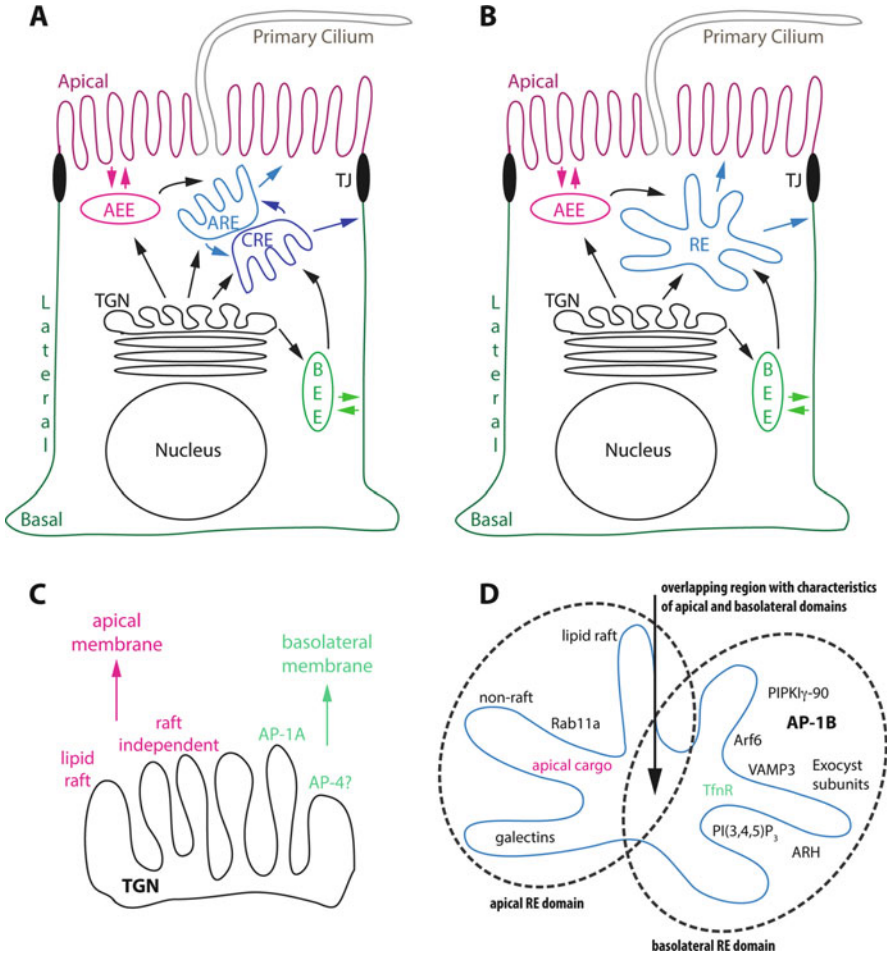

Fig. 7.1 This model figure depicts the intracellular sorting stations present in polarized epithelial cells. (**a**) illustrates the model of two separated populations of REs, whereas (**b**) illustrates the model championing only one RE. (**c**) depicts an expanded version of the TGN showing the different TGN domains responsible for apical and basolateral sorting. (**d**) depicts an expanded version of the RE in (**b**) showing its subdomains. Note that the overlapping region between the apical and the basolateral sorting domain may contain any of the components listed for each domain including, but not necessarily limited to, Rab11a, Arf6, and exocyst subunits. See Sect. 7.3.4 for a detailed description of the basolateral RE domain

surface is further complicated by the observation that many proteins contain multiple signals of varying strengths. Thus, mutation or ablation of a given signal on a protein does not always lead to its non-polarized distribution as one would expect, but rather can result in its profound retargeting to the opposing plasma membrane domain or to another compartment. These difficulties aside, we are slowly expanding our understanding of the specific signals that direct proteins apically and how these signals are interpreted by the cellular machinery.

7.2.1 Apical Sorting Signals

The apical sorting information on proteins described to date constitutes a remarkably diverse collection of signals that includes peptide sequences, posttranslational modifications, and structural information [reviewed in (Weisz and Rodriguez-Boulan 2009)].

7.2.2 GPI Anchors

The first known apical targeting signal to be identified was the presence of a glycosylphosphatidylinositol (GPI) lipid anchor on proteins expressed in MDCK cells (Lisanti et al. 1989). These anchors enable protein localization to glycolipid-enriched lipid microdomains (lipid rafts), and it was proposed that this in turn facilitates their segregation into apically destined sorting platforms at the TGN and possibly at distal sites along the biosynthetic pathway as well (Simons and Ikonen 1997). However, subsequent studies have shown that this is an overly simplistic model. There is now considerable evidence that additional clustering of GPI-anchored proteins (GPI-APs) into high-molecular-weight oligomers is required for apical sorting [reviewed in (Muniz and Zurzolo 2014)]. This clustering may be mediated by protein-protein, protein-lipid, and/or glycan-dependent interactions and may be cell type dependent (Imjeti et al. 2011; Paladino et al. 2004, 2008). Remodeling of GPI-AP acyl chains can also facilitate oligomer formation (Muniz and Zurzolo 2014).

7.2.3 Glycan-Dependent Sorting Signals

A second class of apical sorting signals is dependent on the addition or modification of N- or O-linked oligosaccharides [reviewed in (Potter et al. 2006a)]. Dissection of these signals can be difficult as N-glycans are often essential for the proper folding and ER export of proteins. That said, specific N-glycan structures or linkages have directly been implicated in apical sorting of several proteins. For example, sialylation of N-glycans on the sialomucin endolyn has been demonstrated to be essential for its efficient apical sorting (Mo et al. 2012).

The paucity of reagents and approaches to perturb O-linked glycans has made the role of this modification more challenging to address. Specifically, it has been difficult to determine whether the oligosaccharides themselves, as opposed to the structural consequences of O-glycosylation at a specific region of a protein, is essential for polarized sorting (Yeaman et al. 1997; Youker et al. 2013). For example, the O-glycosylated imperfect tandem repeats of MUC1 contain

transferable apical sorting information, but no specific sugar residues have yet been shown to be essential for sorting (Kinlough et al. 2011).

How glycans function to direct sorting remains unclear, although there is increasing evidence for a role of galectins in mediating the apical sorting of a subset of proteins (Delacour et al. 2009). Galectins 3 and 9 have been shown to function in apical sorting of cargos that do not partition into lipid rafts in MDCK cells (Delacour et al. 2006; Mo et al. 2012). Similar to the clustering mechanism proposed for lipid raft-associated proteins, galectin 3-mediated oligomerization of apical cargos into high-molecular-weight complexes has been demonstrated (Delacour et al. 2007). Interestingly, galectins 4 and 9 also bind to glycolipids in lipid rafts in intestinal and kidney cells, respectively, and may also facilitate the clustering of raft-associated proteins (Delacour et al. 2005; Mishra et al. 2010; Stechly et al. 2009).

7.2.4 Peptide-Based Sorting Signals

Peptide sequences present in either the luminal, transmembrane, or cytoplasmically exposed regions of many proteins have been identified as important for their apical localization. Indeed, peptide-based signals are the most common type of sorting information identified for polytopic proteins, including many ion transporters and G-protein-coupled receptors. However, no unifying mechanism has emerged for how these sequences function to direct apical localization. Rather, these identified motifs have been variously implicated in polarized sorting, surface delivery, and cell surface retention. Sorting of proteins via selective retention at the plasma membrane will be discussed in more detail in Sect. 7.4.

The observation that some splice variants of ion transporters are targeted to opposing domains of epithelial cells has provided a unique opportunity to identify apical and basolateral sorting information encoded within these proteins. For example, in the presence of a 45-amino-acid insertion in the first cytosolic loop of the Ca^{2+}-ATPase, PMCA2 directs apical sorting, whereas splice variants lacking this sequence are targeted basolaterally in MDCK cells (Chicka and Strehler 2003). While this example might imply that the determination of peptide-based apical sorting information is straightforward, this is far from the case. Indeed, there are only a few cases where linear amino acid sequences that confer transferable apical localization have been demonstrated, and even then, the identification of key residues required for sorting has been fraught. For example, the sole difference between the basolaterally targeted human GLUT9a and the apically targeted splice variant GLUT9b is the absence of 29 amino acids within the amino terminus of GLUT9b. The shorter amino terminus of GLUT9b could redirect the normally basolaterally trafficked GLUT1 transporter to the apical surface, demonstrating that it contains transferable apical sorting information. However, no specific sequence unique to GLUT9b could be identified as essential for apical sorting (Bibee et al. 2013). One possible conclusion of these studies is that the amino terminal

domain of GLUT9b interacts with or positions other regions of the transporter to create a conformation that somehow promotes apical sorting. These observations are consistent with a model in which apical sorting of some proteins is conferred by information dependent on a specific protein conformation as opposed to a specific amino acid sequence.

For other ion transporters, short stretches of amino acids have been identified most frequently within cytosolically oriented loops or tails, which direct their apical localization. A ten-amino-acid sequence (PIKPVFKGFS) that includes a putative β-turn within the C-terminus of the human sodium-dependent vitamin C transporter hSVCT1 is required for apical delivery in MDCK cells (Subramanian et al. 2004). Interestingly, similar motifs have also been shown to be important for apical targeting of the neuronal sodium-dependent glutamate transporter EAAT3 (Cheng et al. 2002) and in the rat sodium-dependent bile acid transporter (Sun et al. 2003). In addition, short and apparently unrelated peptide sequences direct the apical localization of the potassium MaxiK channel (Kwon and Guggino 2004), the mouse sodium-phosphate cotransporter NaPi-IIc [WLHSL; (Ito et al. 2010)], and the cystic fibrosis transmembrane conductance regulator [CFTR; (Milewski et al. 2000)]. It is not yet known how these nonterminal short sequences control apical polarity. One possibility is that they interact directly with apical targeting or retention machinery. In support of this, a five-amino-acid (SQDAL) sequence within a cytoplasmic loop of the ATP-binding cassette transporter isoform C2 (ABCC2; aka MRP2) is required for apical sorting in HepG2 cells via an apparently saturable mechanism (Bandler et al. 2008; Emi et al. 2012).

Despite the sizable literature on polarized sorting, we still know little about the mechanisms by which apical sorting determinants on polytopic proteins, including ion transporters, are interpreted. The difficulty in identifying specific sequences or transferable motifs that confer apical sorting of polytopic proteins may in part be due to the intrinsic challenges of generating mutations that do not disrupt their folding or interactions with accessory subunits. Alternatively, the sequences or domains required for apical sorting may influence targeting via steric or conformational effects rather than by binding to interacting proteins. Consistent with this idea, there are only a very few cases where apical sorting information has been demonstrated to be saturable or where similar peptide motifs have been identified in multiple proteins. Once proteins arrive at the apical surface, binding to the cytoskeleton via scaffolding proteins or PDZ-domain interactions can enhance their retention at this domain (see Sect. 7.4 for further details).

7.2.5 Apical Sorting Mechanisms

Clustering mediated by incorporation into glycolipid-enriched microdomains or by galectin-mediated cross-linking is implicated in the polarized sorting of lipid-raft-associated and glycan-dependent apical proteins. The possibility that peptide-based sorting determinants regulate the conformation of polytopic proteins suggests that

clustering may be an underlying mechanism for the apical targeting of this class of proteins as well (Bigay and Antonny 2012; Dehmelt and Bastiaens 2010). This is not to say that other features of polytopic proteins do not contribute to their clustering and/or apical sorting. There is certainly precedence for incorporation of ion channels and transporters into lipid rafts (Ares and Ortiz 2012; Bravo-Zehnder et al. 2000; Chen et al. 2011; Hill et al. 2007; Riquier et al. 2009). Moreover, glycan processing may enhance the oligomerization of polytopic proteins (Ozaslan et al. 2003). Thus, various features of a given protein may guide its incorporation into homomeric or heteromeric arrays that facilitate apical delivery.

How might cargo clustering lead to apical sorting? Cytosolic domains of some apical proteins may bind directly to Rab proteins, molecular motors, or adaptors. Proteins that may facilitate clustering and/or trafficking to the apical membrane include but are not limited to VIP17/MAL, caveolin-1, annexins, and the four-phosphate adaptor protein 2 [FAPP2; reviewed in (Fölsch 2008)]. Clustering will increase the avidity of these interactions to create a sorting "platform" that may nucleate the assembly of an apically destined transport carrier. Many apically destined proteins have very short (<10 amino acid) cytoplasmic tails or, in the case of GPI-anchored proteins, no domains accessible to the cytosol. Co-clustering of these proteins with others that can recruit sorting machinery may be essential for their sorting. Moreover, incorporation of transmembrane VAMPs into these clusters may be necessary for eventual fusion of transport carriers with the apical surface. VAMPs implied in fusion of vesicles with the apical membrane are VAMP2, VAMP7 (aka TI-VAMP), and VAMP8 (Caceres et al. 2014; Galli et al. 1998; Pocard et al. 2007). It should be noted that the concept of spatially regulated protein self-assembly or scaffolding into higher-order clusters is not unique to apical sorting, and indeed models for self-organization have been demonstrated to drive numerous cellular functions [elegantly reviewed in (Bigay and Antonny 2012; Dehmelt and Bastiaens 2010)].

Another unresolved question is: where along the biosynthetic pathway does apical sorting occur? Newly synthesized apical and basolateral proteins are segregated at least by the time they leave the TGN. Indeed, there is also evidence that raft-associated and raft-independent apical proteins are segregated when leaving this compartment [Fig. 7.1c, (Guerriero et al. 2008; Jacob and Naim 2001)]. Moreover, it has become increasingly clear that these two classes of proteins take distinct routes to the apical surface and that these routes intersect different endocytic compartments (Cresawn et al. 2007; Fölsch et al. 2009). Lipid raft-associated proteins may pass through AEEs, whereas glycan-dependent and some other apical cargos transit Rab11a-positive AREs. In addition, intestinal and kidney cells also express the Rab11b isoform, which appears to be localized to a subdomain of the ARE (Lapierre et al. 2003). Apically recycling CFTR and the epithelial sodium channel ENaC traffic through Rab11b-positive endosomes, although it is not known whether newly synthesized proteins also access this compartment (Butterworth et al. 2012; Silvis et al. 2009). Complicating matters further, there are multiple exit routes for proteins that traffic through the ARE (Mattila et al. 2012).

How can the concept of cluster-dependent sorting be reconciled with the presence of multiple apical sorting stations? If there is continual segregation of apical proteins into different export pathways as they move from one compartment to another, there may be different subsets of clusters that form at each site. Indeed, clustering mediated by distinct mechanisms may be initiated in different compartments. It is believed that proteins are incorporated into lipid rafts during their transit through the Golgi (Guerriero et al. 2008; Jacob and Naim 2001), and galectin 9 that binds to the raft-enriched glycolipid Forssman antigen (as well as to non-raft-associated proteins) appears to access these structures at the TGN of MDCK cells (Mishra et al. 2010). In contrast, galectin 3 interaction with glycosylated proteins occurs after they leave the TGN (Delacour et al. 2007; Straube et al. 2013). Thus, non-raft-associated proteins that traffic together to the ARE (e.g., proteins with glycan-dependent and peptide-based sorting signals) might be segregated into distinct populations that differentially exit that compartment based on their ability to interact with galectin 3. Whether such hierarchical arrays of clustering actually occur remains to be determined.

7.3 Sorting to the Basolateral Membrane

Like apical targeting, there are multiple pathways that cargos may follow to reach the basolateral membrane during biosynthetic delivery and endocytic recycling (Arnspang et al. 2013; Farr et al. 2009; Fölsch et al. 2009). Many newly synthesized cargos travel from the TGN into REs during biosynthetic delivery, and cargos may be directed into the basolateral pathway from either of these sites (Ang et al. 2004; Fölsch et al. 2009; Nokes et al. 2008). Often, basolateral sorting signals are linear peptide motifs that are *cis*-dominant over apical sorting information such that mutation of the basolateral sorting signals results in a protein that is sorted to the apical membrane instead. In addition, basolateral sorting signals are often colinear with lysosomal sorting signals and endocytic motifs. Indeed, low amounts of the lysosomal-associated membrane glycoprotein Lamp1 and the cation-dependent mannose 6-phosphate receptor (CD-MPR) that cycles between the TGN and late endosomes exclusively cycle through the basolateral membrane in MDCK cells (Distel et al. 1998; Hunziker et al. 1991; Nabi et al. 1991).

7.3.1 Basolateral Sorting Signals and Adaptors

Linear peptide sorting motifs that target proteins to the basolateral membrane are frequently either tyrosine based [YxxØ, where Ø is a bulky hydrophobic amino acid (F, I, L, M, or V), and FxNPxY] or dileucine based (LL, LI, or LM) often in the form of [D/E]xxxL[L/I]. These signals are generally recognized by cytosolic adaptor complexes, most commonly heterotetrameric clathrin adaptor protein

(AP) complexes AP-1 (γ-β1-μ1-σ1), AP-2 (α-β2-μ2-σ2), AP-3 (δ-β3-μ3-σ3), and AP-4 (ε-β4-μ4-σ4) that does not associate with clathrin (Boehm and Bonifacino 2001; Hirst and Robinson 1998; Ohno et al. 1999). YxxØ signals are recognized by the AP medium subunits, whereas [D/E]xxxL[L/I]-signals are recognized simultaneously by σ1-γ – subunits of AP-1 as well as σ2-α of AP-2 and σ3-δ of AP-3 (Doray et al. 2007; Janvier et al. 2003; Mattera et al. 2011). Another form of dileucine signal (DxxLL) is recognized at the TGN by monomeric clathrin adaptor proteins of the GGA (Golgi-localized, γ-ear containing, Arf-binding) protein family [discussed in (Kirchhausen 2002)], perhaps leading to incorporation into AP-1A vesicles (Doray et al. 2002). Moreover, the cytoplasmic tail of the transforming growth factor α (TGFα) encodes a specialized LL signal that is aided by a cluster of negative charges and recognized by the cytosolic adaptor Naked2 for sorting to the basolateral membrane (Li et al. 2004, 2007). Finally, FxNPxY signals are bridged to clathrin adaptor complexes by connector proteins such as numb, Dab2, and autosomal recessive hypercholesterolemia protein (ARH) [reviewed in (Traub 2003)].

Other peptide-based sorting motifs have been described that seem to be far less abundant. For example, the basolateral sorting signal of CD147 consists of only a single leucine residue (Deora et al. 2004). A different, non-related mono-leucine signal for basolateral sorting was also discovered in amphiregulin (Gephart et al. 2011). No adaptor proteins have yet been described that recognize these mono-leucine sorting signals. In addition, proteins may be sorted to and selectively retained at the basolateral membrane by means of PDZ-binding motif interaction with PDZ domain-containing proteins [reviewed in (Brone and Eggermont 2005); see also Sect. 7.4]. PDZ-directed sorting from endosomes may be facilitated by the sorting nexin 27 [SNX27; (Lauffer et al. 2010)]. Furthermore, basolateral sorting from early endosomes may be facilitated by the "retromer" complex and SNX17 [reviewed in (Bonifacino 2014)]. SXN17 binds NPxY peptide motifs (Donoso et al. 2009; Stiegler et al. 2014).

Epithelial cells express two closely related AP-1 complexes, AP-1A and AP-1B, that differ only in the incorporation of their respective medium subunits μ1A or μ1B. Notably, μ1B expression is specific to columnar epithelial cells including MDCK and Caco-2 cell lines (Fölsch et al. 1999; Ohno et al. 1999). So far both AP-1A and AP-4 have been implicated in sorting of basolateral cargos at the TGN (Fig. 7.1c), and AP-1B was shown to facilitate cargo sorting in REs [reviewed in (Ang and Fölsch 2012; Rodriguez-Boulan et al. 2013)]. Interestingly, whereas the connector proteins numb and Dab2 specifically interact with AP-2, ARH also interacts with AP-1B in REs to facilitate basolateral sorting of cargos with FxNPxY signals (Kang and Fölsch 2011).

7.3.2 AP-1B Expression in the Kidneys

AP-1B is expressed in many regions of the kidney including the medullary and cortical thick ascending limbs (TALs) and the cortical collecting duct (CCD) (Schreiner et al. 2010). However, AP-1B is not expressed in the proximal tubules and cell lines that were derived from that region (e.g., LLC-PK1 cells) (Ohno et al. 1999; Schreiner et al. 2010). Therefore, LLC-PK1 cells are a useful model system for analyzing AP-1B function and to determine if a protein depends on AP-1B for steady-state localization at the basolateral membrane. To this end researchers use LLC-PK1 cells stably expressing exogenous copies of μ1B or μ1A as a control. Proteins that are apical in μ1A-expressing LLC-PK1 cells, but basolateral in μ1B-expressing cells, are then identified as AP-1B-dependent cargos (Fields et al. 2007; Fölsch et al. 1999). Because AP-1B is the only adaptor complex that localizes in REs to facilitate basolateral sorting during endocytic recycling (Fölsch et al. 1999, 2001; Gan et al. 2002), its cargo proteins will be missorted to the apical membrane in the absence of functional AP-1B (Fields et al. 2007; Fölsch et al. 1999; Gravotta et al. 2007; Sugimoto et al. 2002). Proteins dependent on AP-1B for basolateral localization include many nutrient receptors like low-density lipoprotein receptor (LDLR) and TfnR, as well as the adhesion proteins E-cadherin and β1 integrin, and the growth hormone receptor EGFR (Fölsch et al. 1999; Gravotta et al. 2007; Ling et al. 2007; Ryan et al. 2010).

7.3.3 ARH Expression in the Kidneys

ARH expression is found predominantly in the distal nephron tubules including the distal convoluted tubule (DCT), the connecting tubule (CNT), as well as the cortical collecting duct (CCD) and to a lesser extent in the regions of the thick ascending limbs (TALs) (Fang et al. 2009). Thus, ARH expression largely overlaps with AP-1B expression in the kidneys ensuring AP-1B-dependent cargo sorting of proteins with FxNPxY sorting motifs.

7.3.4 Mechanisms of AP-1B-Mediated Basolateral Sorting

What is known so far about AP-1B function on a molecular level? It is thought that recruitment of AP-1B onto REs depends on PI(3,4,5)P_3 and possibly Arf6 (Fields et al. 2010; Shteyn et al. 2011). Strikingly, PI(3,4,5)P_3 accumulates in REs only in epithelial cells that express AP-1B in a reaction that may involve the phosphatidylinositol 4-phosphate 5-kinase PIPKIγ-90 (Fields et al. 2010). Indeed PIPKIγ-90 was shown to directly interact with AP-1B, but not AP-1A, and to play a role in basolateral sorting of E-cadherin (Ling et al. 2007). After membrane recruitment,

AP-1B selects its cargos either directly or with the help of its co-adaptor ARH (Kang and Fölsch 2011). In addition, AP-1B recruits at least 2 subunits of the exocyst complex to REs for incorporation into AP-1B vesicles (Fölsch et al. 2003). AP-1B vesicles also contain the SNARE protein VAMP3 (aka cellubrevin) (Fields et al. 2007), and disruption of VAMP3 function in MDCK cells leads to dispersed REs to which AP-1B no longer localizes (Fields et al. 2007).

Tethering of AP-1B vesicles to the basolateral membrane is thought to depend on the exocyst complex (Grindstaff et al. 1998; Yeaman et al. 2001), and fusion of the vesicles may involve VAMP3 on the vesicles and syntaxin 4 at the basolateral membrane (Fields et al. 2007). Interestingly, basolateral sorting of syntaxin 4 itself depends on AP-1B expression, although how AP-1B influences basolateral sorting of syntaxin 4 is not yet entirely clear (Reales et al. 2011; Torres et al. 2011).

In addition to AP-1B-dependent sorting, syntaxin 4 is most likely also involved in fusion of vesicles that originated from BEEs. Indeed, syntaxin 4 was shown to directly interact with the early endosomal Rab4 protein (Li et al. 2001), and disruption of Rab4 function in MDCK cells resulted in apical missorting of basolateral membrane proteins (Mohrmann et al. 2002).

7.3.5 Basolateral Sorting of Transporters

Many transporters at the basolateral membrane contain tyrosine-based sorting motifs, but are independent of AP-1B. This is perhaps due to low internalization rates and/or stable retention at the basolateral membrane. For example, the kidney anion exchanger 1 (kAE1) contains a YDEV signal in its cytoplasmic tail that is needed for AP-1A-mediated basolateral sorting at the TGN (Almomani et al. 2012; Junking et al. 2014; Toye et al. 2004). However, although kAE1 is initially sorted to the basolateral membrane independent of AP-1B expression, AP-1B is probably needed during endocytic recycling (Almomani et al. 2012). Tyrosine-based motifs have also been described in the chicken kAE1-4 splice isoform that contains these signals in a 63-amino-acid N-terminal extension, which is missing in the apically sorted kAE1-3 splice isoform (Adair-Kirk et al. 1999). Interestingly, a disease-causing mutation in the C-terminal residue (M909T) of the human kAE1 results in the creation of a type I PDZ ligand and partial redirection of the protein to the apical surface (Fry et al. 2012). Because the wild-type protein is targeted directly to the basolateral domain, it was concluded that the presence of this PDZ-interacting motif causes apical misdirection of a subset of the mutant kAE1 within the biosynthetic pathway (Fry et al. 2012), although it is also possible that this mutation causes selective retention at the apical membrane.

Other transporters that do not rely on AP-1B for basolateral targeting are the homotetrameric water channels aquaporins 3 and 4 that are expressed in renal collecting ducts. Aquaporins have six transmembrane domains, and both the N- and C-termini are exposed to the cytosol. Aquaporin 3 contains a tyrosine-based sorting motif that overlaps with a dileucine-based signal (YRLL) in its N-terminus

(Rai et al. 2006). Mutation of either signal partially redirected aquaporin 3 to the apical membrane, and mutation of both motifs effectively disrupted surface delivery. Because aquaporin 3 is also localized to the basolateral membrane in LLC-PK1 cells, it was concluded that AP-1B is not needed for its basolateral sorting (Rai et al. 2006). Indeed, other receptors that contain dileucine-based motifs such as the Fc receptor FcRII-B2 likewise do not need AP-1B for basolateral targeting (Fields et al. 2007).

In contrast to aquaporin 3, aquaporin 4 contains basolateral sorting information in its C-terminus (Madrid et al. 2001). The first of two motifs is a tyrosine-based (S)YMEV(E) motif that also serves as an internalization motif and directly interacts with the medium subunits of AP-2 and AP-3, but not with AP-1A. The second motif is an acidic dileucine motif (ETEDLIL). Curiously, the YMEVE sequence may conform to the consensus sequence recognized by AP-4 (Burgos et al. 2010; Dell'Angelica et al. 1999), and it remains to be determined if it indeed interacts with this adaptor. Importantly, this motif overlaps with a casein kinase II (CKII) consensus site (SxxE/D), and phosphorylation by CKII introduces a negative charge directly upstream of the tyrosine leading to enhanced interaction with AP-3 and thus delivery of aquaporin 4 into lysosomes for degradation (Madrid et al. 2001). It is tempting to speculate that aquaporin 4 may be sorted at the TGN into AP-4 vesicles for basolateral targeting. After internalization, interaction with AP-3 may effectively remove the water channel from BEEs such that little if any aquaporin 4 may enter REs. Indeed, aquaporin 4 is also localized to the basolateral membrane in renal proximal tubules, indicating that AP-1B plays no role in its basolateral sorting (van Hoek et al. 2000).

7.4 Retention at the Cell Surface Through Interaction with PDZ Domains

Selective retention at the apical or basolateral membrane may be achieved through interaction with a specialized cytoskeleton. For example, ankyrin G and β2 spectrin specifically anchor proteins at the lateral membrane (Kizhatil et al. 2007). More common is retention through interaction of PDZ-binding motifs at the extreme C-terminus of transmembrane cargos with PDZ domains in scaffolding proteins. However, the presence of a PDZ-binding motif does not assure that it functions in retention, as disruption of the C-terminal PDZ-interacting sequences of hSMVT or ABCC2 has no effect on their localization (Nies et al. 2002; Subramanian et al. 2009). In other cases, retention at the apical membrane through a PDZ-binding motif may be overridden by a strong basolateral sorting signal as was the case for aquaporin 2 chimeras with aquaporin 3, in which the apical targeting signal in aquaporin 2, which is a PDZ-binding motif, was maintained, and the basolateral sorting information of aquaporin 3 was added to the N-terminus effectively directing the chimera to the basolateral surface (Rai et al. 2006).

Although PDZ-binding motifs are thought to primarily facilitate selective retention at the plasma membrane, they are often also important for surface delivery (Lauffer et al. 2010; Maday et al. 2008).

PDZ-binding motifs may be present in addition to other sorting signals. For example, the basolateral potassium channel Kir2.3 contains a type I PDZ-binding motif that partially overlaps with a basolateral sorting signal; both motifs are needed for robust basolateral localization of Kir2.3 (Le Maout et al. 2001). Importantly, the PDZ-binding motif is necessary for stable expression at the plasma membrane, and its deletion results in largely intracellular localization of the mutant channel. Retention at the basolateral membrane is most likely achieved through binding of Kir2.3 to the PDZ domain containing Lin-7/CASK protein complex (Olsen et al. 2002). Similarly, the epithelial γ-aminobutyric acid (GABA) transporter (BGT-1) is also retained at the basolateral membrane through interactions with Lin-7 (Perego et al. 1999). In MDCK cells, Lin-7 associates with adherens junctions through interaction with β-catenin (Perego et al. 2000).

Some transporters have tyrosine-based signals that are used for internalization, but not for basolateral targeting. For example, the renal outer medullary potassium secretory channel ROMK (aka Kir1.1) has an YDNPNFV motif that binds to ARH and serves as an endocytic signal (Fang et al. 2009). However, ROMK is localized to the apical membrane domain, although it is expressed in the kidney distal tubules that express both ARH and AP-1B (Fang et al. 2009; Schreiner et al. 2010). ROMK has a PDZ-binding motif at its C-terminus that is necessary for membrane expression of ROMK and interacts with the PDZ domain-containing proteins Na/H exchange regulatory factors NHERF1 and 2 (Yoo et al. 2004). Perhaps ROMK binding to NEHRF selectively stabilizes ROMK at the apical membrane effectively overriding basolateral sorting by AP-1B and ARH. PDZ-binding motifs have also been shown to contribute to the steady-state apical localization of other ion transporters (Brone and Eggermont 2005) including CFTR (Milewski et al. 2000; Moyer et al. 1999), TRPV4 and TRPV5 (van de Graaf et al. 2006), and PMCA2b (Antalffy et al. 2012).

Interestingly, stabilizing proteins at the apical membrane through binding to NHERF may also ensure their exclusion from the primary cilium that extends from the apical membrane. Primary cilia are important appendages needed for correct cell signaling and homeostasis, and their membrane composition is distinct from the apical (and basolateral) membranes. Most apical membrane proteins are excluded from the primary cilium, and it is thought that the base of the cilium contains a diffusion barrier that is formed by a member of the septin GTPase family, septin 2 (Hu et al. 2010). However, additional mechanisms exist. For example, it has been shown that interaction with NHERF1 prevents the apical membrane protein podocalyxin from entering the primary cilium (Francis et al. 2011). It is thought that NHERF1 immobilizes proteins in the apical membrane by cross-linking them to the apical actin network via ERM family members (Francis et al. 2011). It seems reasonable to assume that other apical proteins that interact with NHERF will be excluded from the primary cilium as well.

7.5 Sorting of Multi-subunit Transporters

Polarized sorting of heteromeric transporters may appear convoluted as each individual subunit may contain sorting information. Moreover, association with different subunit isoforms may drive the heteromers toward the apical or basolateral membrane. A well-studied example of this is the Na,K-ATPase, which in most tissues is localized basolaterally. The Na,K-ATPase consists of three subunits: the catalytic α-subunit, the β1-subunit that is thought to aid TGN exit of the complex, and the γ-subunit that modulates ion channel activity. Multiple sorting signals have been described for these subunits. For example, both the α-subunit and β1-subunit contain basolateral sorting information (Marrs et al. 1995; Muth et al. 1998; Shoshani et al. 2005). However, the signal in the α-subunit can be overturned by pairing with a different β-subunit (Vagin et al. 2005). Whereas expression of β1 directs the Na,K-ATPase α-subunit to the basolateral surface, β2 is responsible for apical localization of the Na,K-ATPase in gastric adenocarcinoma cells. Interestingly, this apical distribution of β2 is apparently mediated by its prevalence of N-glycans, although the mechanism is unknown. Moreover, addition of new N-glycosylation consensus sequences to the more sparsely glycosylated β1-subunit converts it to an apically localized protein (Vagin et al. 2005). Basolateral localization of the Na,K-ATPase has been further attributed to selective retention via interaction with the cytoskeleton (i.e., ankyrin G) (Kizhatil et al. 2007; Marrs et al. 1995) or through homotopic interaction of β1-subunits across adjacent cells (Shoshani et al. 2005). Interestingly though, basolateral localization of the β1-subunit in sensory hair cells in zebrafish depends on AP-1 expression (Clemens Grisham et al. 2013), indicating that at least the β1-subunit may have a tyrosine or dileucine-based motif that could be recognized by AP-1A at the TGN for basolateral sorting. Notably, AP-1B is not necessary for the basolateral localization of the Na,K-ATPase (Fölsch et al. 1999).

Both AP-1A and AP-1B have been implicated in the basolateral sorting of the H,K-ATPase β-subunit. This subunit has a reversed tyrosine-based signal, FRHY, that interacts with AP-1A and AP-1B in vitro (Duffield et al. 2004). The pump is localized to the basolateral membrane in MDCK cells and to the apical membrane in LLC-PK1 cells (Roush et al. 1998). Curiously though, the pump is also localized to the apical membrane in LLC-PK1 cells stably transfected to express AP-1B, which normally reinstates basolateral sorting of proteins with tyrosine-based sorting motifs. This suggests that the FRHY signal may be masked in LLC-PK1 cells by an unknown mechanism (Duffield et al. 2004).

Caplan and colleagues have employed the sequence homology and structural similarities between the apically targeted gastric H,K-ATPase and the basolateral Na,K-ATPase to dissect the apical targeting information in the former. Interestingly, they found that replacing the fourth transmembrane domain (TM4) of the Na,K-ATPase α-subunit with that of the closely related H,K-ATPase sequence was sufficient to redirect the Na,K-ATPase to the apical surface of polarized LLC-PK1 cells (Dunbar et al. 2000). In addition, apical targeting of the Na,K-ATPase was

also observed when only the cytoplasmic and intracellular loop sequences surrounding this domain (but not TM4 itself) were replaced with the corresponding sequences from the H,K-ATPase. Similarly, a six-amino-acid sequence within the extracytoplasmic loop preceding TM4 of the related non-gastric H,K-ATPase was shown to be necessary for apical targeting in MDCK cells. In this case, a single point mutation in this region was sufficient to disrupt apical sorting (Lerner et al. 2006).

7.6 Challenges to the Field

Despite the many advances that were achieved over the years in determining polarized sorting mechanisms in epithelial cells, there remain many challenges to researchers in the field. For example, a confounding problem when working with ion transporters is that they are generally expressed at low levels in cells, and their folding, assembly, and surface delivery are often inefficient. Due to these technical challenges, polarized sorting determinants of polytopic proteins are often dissected by monitoring the steady-state distribution of heterologously expressed and sometimes epitope-tagged mutants. Unfortunately, by using this approach, it is often difficult to gather information about the mechanisms that enable polarized localization of a given protein (e.g., polarized biosynthetic targeting vs. surface retention).

Another challenge for the field is the lack of available well-differentiated cell culture models to study the trafficking of ion transporters in their native cell type. As noted above, polarized delivery routes differ significantly between cells of liver, intestinal, and kidney origin. Moreover, there can be differences in the regulation of membrane traffic between distinct cell types in a single organ. As one example, cells in the proximal tubule of the kidney are highly specialized for apical endocytosis as opposed to cells further downstream along the nephron, and the organization of apical endocytic compartments in these cells reflects the functional elaboration of this pathway (Mattila et al. 2014). Similarly, the absence of μ1B expression in the proximal tubules has profound implications for the localization of many proteins. However, few cell culture models are available that maintain both the expression of endogenous transporters and the polarization of distinct kidney nephron cell types. While many challenges remain, recent advances in protein expression and knockdown strategies as well as the development of live animal imaging modalities have facilitated the study of ion transporter trafficking in situ. These techniques, in combination with the use of MDCK cells as an in vitro model, should lead to a better understanding of surface delivery of ion transporters in the future.

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