The subapical compartment: a traffic center in membrane polarity development

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Summary

Spatially separated apical and basolateral plasma membrane domains that have distinct functions and molecular compositions are a characteristic feature of epithelial cell polarity. The subapical compartment (SAC), also known as the common endosome (CE), where endocytic pathways from both surfaces merge, plays a crucial role in the maintenance and probably the biogenesis of these distinct membrane domains. Although differences in morphology are apparent, the same principal features of a SAC can be distinguished in different types of epithelial cells. As polarity develops, the compartment acquires several distinct machineries that, in conjunction with the cytoskeleton, are necessary for polarized trafficking. Disrupting trafficking via the SAC and hence bypassing its sorting machinery, as occurs upon actin depolymerization, leads to mis-sorting of apical and basolateral molecules, thereby compromising the development of polarity. The structural and functional integrity of the compartment in part depends on microtubules. Moreover, the acquisition of a particular set of Rab proteins, including Rab11 and Rab3, appears to be crucial in regulating molecular sorting and vesicular transport relevant both to recycling to either plasma membrane domain and to de novo assembly of the apical domain. Furthermore, subcompartmentalization of the SAC appears to be key to its various functions.

Key words: Subapical compartment, SAC, Endocytosis, Polarity, Trafficking

Introduction

Polarity is a ubiquitous feature of the biology of eukaryotic cells and entails the establishment and maintenance of spatial and functional asymmetry, triggered by cues from the external and intracellular environments. For example, cell movement in response to chemoattractants causes asymmetric shape changes, generating a leading edge that protrudes at the front of the cell and a rear edge that retracts (Lauffenburger and Horwitz, 1996). Instrumental in this is the establishment of membrane polarity. Molecules such as (glyco)sphingolipids segregate to the two opposing membrane surfaces (Gomez-Mouton et al., 2001) and, by virtue of their ability to organize microdomains, may establish a platform for recruitment of the different molecular machineries required at these locations. Polarity is also apparent in neuronal cells, in which membrane domains are segregated by a diffusion barrier formed by a row of protein ‘pickets’ assembled on a membrane-cytoskeletal meshwork at the axonal initial segment (Nakada et al., 2003). However, most attention has focused on epithelial cells and it is these that perhaps best exemplify the principles of cell polarity, displaying asymmetric cellular morphology, molecular composition and functional properties.

Epithelial cells line many tissues and simultaneously face different environments: the lumen on one side, and adjacent cells and underlying connective tissue on the other. Accordingly, compartmentalization of the cell surface and hence the operation of distinctly localized sorting and retention mechanisms are crucial for their function. Signals triggered by extracellular matrix and cell-cell cues, and relayed by key players such as integrins and E-cadherin–catenin complexes, respectively, cause the establishment of specific intercellular junctions, rearrange the cytoskeletal architecture, and activate sorting mechanisms to generate the so-called apical and basolateral plasma membrane domains (Knust, 2002; Mostov et al., 2003; Nelson, 2003). Tight junctions separate these membrane domains, preventing mixing of membrane proteins and (outer leaflet) lipids between them. Complex signaling and sorting machineries regulate transport to and from either domain. In addition, the apical and basolateral domains communicate by transcytosis, the extent of which depends on the cell type. Here, we briefly summarize what is known about these mechanisms and focus on the role of the subapical compartment (SAC), a subcellular trafficking center that is thought to orchestrate these complex trafficking pathways and is required for the generation and maintenance of epithelial membrane polarity.

Endocytosis and polarity: organization and pathways

Polarization of epithelial Madin-Darby canine kidney (MDCK) cells establishes separate apical and basolateral membrane domains, but also results in a sixfold increase in the surface area of the lateral membrane (Vega-Salas et al., 1987). This demands high levels of membrane biogenesis, which relies on a variety of closely interconnected signaling, biosynthetic, transport and recycling events. Evidently, elements of the endocytic and transcytotic transport pathways are vital in polarity development and maintenance.

In nonpolarized cells, internalization of ligand-receptor complexes by endocytosis proceeds via peripheral early sorting endosomes. From this compartment, receptors might recycle to

the plasma membrane directly, or through the perinuclear recycling endosome, which is localized near the microtubule-organizing center. The dissociated ligands are transferred from early to late endosomes and are delivered to lysosomes for digestion (Sachse et al., 2002). Spatial organization of these complex pathways is partly provided by the cytoskeleton, presumably reflecting the involvement of distinct motor proteins that mediate particular membrane transport steps (Kamal and Goldstein, 2000). For example, anterograde transport from early sorting endosomes, but not direct recycling to the plasma membrane, is inhibited upon disruption of microtubules (Zegers et al., 1998).

The small GTPases Rab4 and 5 are established regulators of early endosomal trafficking. Rab5 is required for the fusion between endocytic vesicles and early endosomes, whereas Rab4 is involved in directing vesicular transport to the recycling endosome, possibly at the level of vesicle assembly and budding (De Wit et al., 2001). Interestingly, Rab-based machineries may cluster in distinct domains and, on early endosomes, distinct microdomains that contain mainly Rab5, Rab5/Rab4, or Rab4/Rab11 have been identified (Sonnichsen et al., 2000; De Renzis et al., 2002). Recycling endosomes are enriched in Rab11 and Rab4 domains (Somsel Rodman and Wandinger-Ness, 2000; Sonnichsen et al., 2000).

Polarized epithelial cells contain distinct populations of basolateral early endosomes (BEEs) and apical early endosomes (AEEs), which do not directly communicate, since neither fluid markers nor apical and basolateral ligand-receptor complexes internalized at either surface intermingle (Huber et al., 2000). The actin cytoskeleton seems to prevent such direct interactions. Indeed, upon latrunculin B-induced depolarization of actin, the distribution of the transferrin receptor (TfR), which normally localizes and recycles to the basolateral surface, becomes randomized, TfR appearing at the apical surface in polarized Caco2 cells (Durrbach et al., 2000) and MDCK cells (Sheff et al., 2002) (see below) By contrast, in untreated cells, traffic moving through the apical and basolateral endosomal populations travels through a common compartment near the apical surface (Fig. 1). This compartment, which seems to be a ubiquitous feature of polarized epithelial cells, is variously termed the apical recycling endosome (ARE) or common endosome (CE) (Mostov et al., 2000), the common recycling endosome (Sheff et al., 2002) or, most commonly in hepatocytes (the major epithelial cells in liver), the SAC (Ihrke et al., 1998; Soroka et al., 1999; van IJzendoorn and Hoekstra, 1999; Rahner et al., 2000).

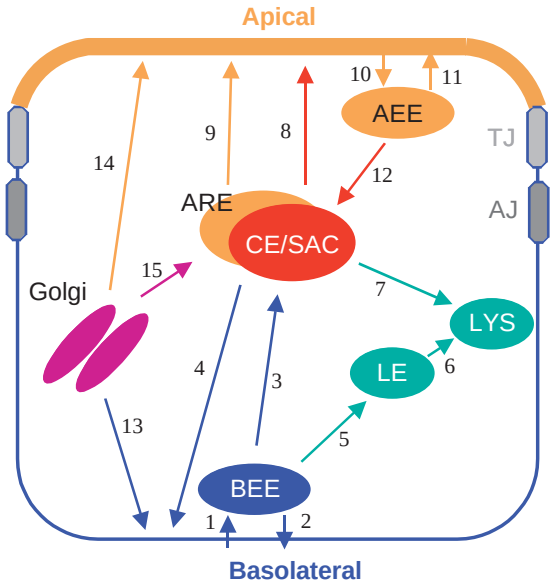

Fig. 1. Membrane trafficking pathways in polarized epithelial cells. Macromolecules, internalized either from the basolateral (1) or apical (10) surface, are delivered to basolateral early endosomes (BEEs) and apical early endosomes (AEEs), respectively. From here, molecules can recycle to the plasma membrane domain of origin (2, 11), or are directed in the degradative, late endosomal (LE)/lysosomal (LYS) pathway (5, 6). Alternatively, basolaterally (3) and apically (12) derived molecules are sorted into a recycling route and meet in a compartment, referred to as the common endosome (CE), subapical compartment (SAC), or apical recycling endosome (ARE). From here, polarized recycling can occur to the basolateral (4) or apical (8) surface. Apical surface targeting can occur directly from the CE/SAC (8), or following a relay from the CE/SAC via the ARE (9) (for details, see text and Fig. 3). From the CE/SAC, proteins can also reach the lysosomes (7). In the biosynthetic pathway, molecules are sorted directly to the basolateral (13) or apical (14) surface. In the latter, some proteins might travel via CE/SAC, prior to their delivery to the apical surface domain (15). AJ, adherens junctions; TJ, tight junctions.

The subapical compartment(s)

Although cell-type-dependent differences in the morphology of the SAC are apparent, its overall appearance is typified by a branching tubulovesicular network that is clustered in the apical region and extends to the cell periphery (Tooze and Hollinshead, 1991; Barroso and Sztul, 1994; Apodaca et al., 1994; Knight et al., 1995; Futter et al., 1998; Rahner et al., 2000; Durrbach et al., 2000; Hansen et al., 2003). The CE identified in MDCK cells is a tubularized endosomal system 60 nm in diameter. Above this is a population of 100-150 nm cup-shaped vesicles immediately underneath the apical membrane, which constitute the ARE (Apodaca et al., 1994; Futter et al., 1998; Gibson et al., 1998). A recent in situ characterization of the SAC in hepatocytes (Fig. 2) similarly revealed the existence of 60-100 nm tubulovesicular structures (analogous to the MDCK CE) and 150-200 nm cup-shaped vesicles in the apical region (analogous to the ARE), through which transcytotic and apical endocytic markers travel sequentially (Rahner et al., 2000).

Ultrastructural studies in MDCK cells and cell fractionation of rat liver suggested that the ARE and tubular CE represent a single compartment that is subdivided into different domains (Futter et al., 1998; Verges et al., 1999). By contrast, the non-overlapping or partially overlapping distribution of distinct SAC-associated markers, such as various Rab proteins, emphasized the subcompartmentalization of the SAC (van IJzendoorn and Hoekstra, 1999). This subcompartmentalization (Fig. 3), as well as the positioning of the SAC around centrosomes, partly depends on microtubules, because there is a selective redistribution of markers following disruption of microtubules. Thus, SAC-localized Mrp2 (Soroka et al., 1999) and Rab11 in hepatocytes (S.C.D.v.IJ. and D.H., unpublished), and ARE-localized Rab11 and Rab25 in MDCK

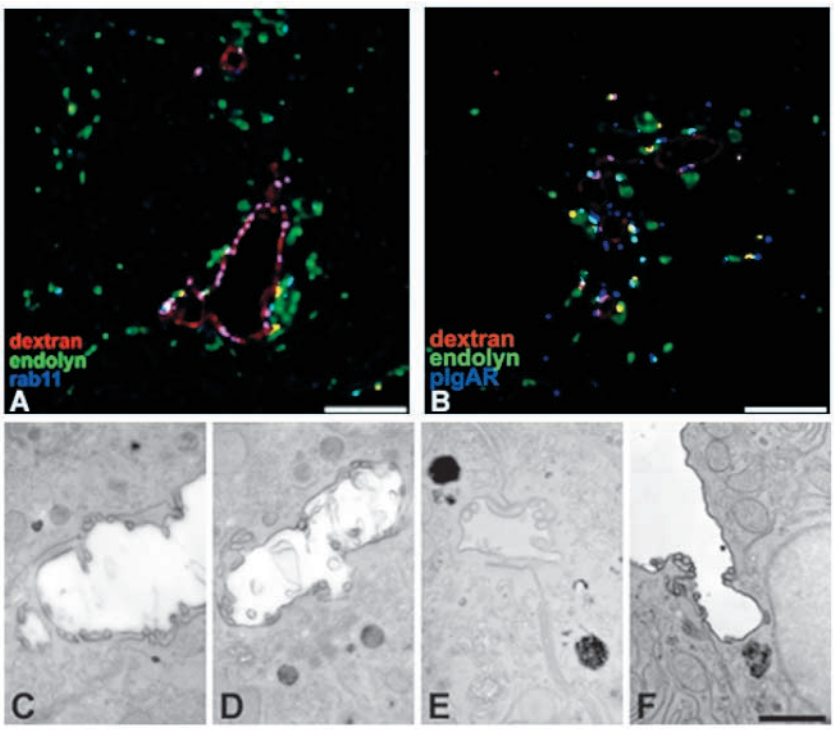

Fig. 2. Visualization of the apical endomembrane system, following internalization of a fluorescent marker by apical endocytosis. (A) Overlap of a marker for apical endocytosis (Texas-Red-labeled dextran), endolyn-78 (green) and Rab11 (blue). Note the overlap of endolyn-78 and Rab11 (cyan) and the overlap of dextran and Rab11 (magenta). Colocalization of all three probes (white) is restricted to the pericanalicular region. Small compartments (<300 nm) are distinguishable close to the apical membrane that contain both endolyn-78 and Rab11, probably reflecting their colocalization in the SAC, as endolyn-78 accumulates in the SAC prior to its delivery to lysosomes. Note that Rab11 is thought to be particularly enriched in the distal part of the SAC or ARE. (B) Overlap of a marker for apical endocytosis with endolyn-78 and basolateral-to-apical transcytosing polymeric IgA receptor (pIgAR). Note the overlap of dextran and endolyn-78 (yellow), of endolyn-78 and pIgAR (cyan), some of which overlaps dextran (white), and of dextran with pIgR-dIgA (magenta). Bars, 5 μm. The apical endocytic pathway (i.e. the route from the AEE to the SAC to lysosomes taken by dextran) intersects the transcytotic route taken by pIgR-dIgA in a small (<300 nm) endolyn-positive SAC. (C-F) Electron micrographs of liver infused retrogradely with HRP for various time intervals. HRP initially localizes to 60-100 nm tubulovesicular structures close to the apical surface (C, 5 minutes). After 10 minutes, multivesicular bodies (MVBs; D) and cup-shaped vesicles (150-200 nm; E) became labeled with HRP, prior to delivery to the lysosomes (F; 15 minutes). Bar, 500 nm. For further details, see text and Rahner et al. (Rahner et al., 2000).

cells (Casanova et al., 1999), redistribute upon nocodazole treatment. By contrast, the distribution of SAC-accumulated sphingolipid analogues [C_6NBD-sphingomylin (SM) or C_6NBD-glucosylceramide (GlcCer)] remains unaltered in HepG2 cells (van IJzendoorn and Hoekstra, 1998), as does that of Rab17 in Eph4 cells [Rab17 regulates basolateral TfR recycling and probably acts more proximally in basoapical transport (Zacchi et al., 1998)]. Note, however, that when stimulated to move into a distal part of the SAC by cyclic (c)AMP signaling, C_6NBD-SM does redistribute in response to nocodazole (Zegers and Hoekstra, 1997; van IJzendoorn and Hoekstra, 2000a), as discussed further below.

HepG2 cells display vesicular structures in the region of the SAC that, at steady state, can be labeled with the fluorescent cholesterol analog dehydroergosterol, but not transferrin or C_6NBD-SM, which both exit basolaterally from the SAC. This also points to the heterogeneous, compartmentalized nature of the SAC (Wüstner et al., 2002). Moreover, in MDCK cells, the ARE but not the CE tubulates in the presence of brefeldin A (BFA), has a higher pH and represents a more distal station in the apical transport pathway (Wang, E. et al., 2000; Brown et al., 2000). Together, the data thus support the idea that the SAC in epithelial cells is subcompartmentalized, rather than constituting a single membrane-bound compartment, and has discrete subdomains. Here, it is useful to distinguish the CE and ARE (Fig. 3). The definition of the latter is somewhat confusing, because the CE itself can recycle cargo from both surfaces, whereas the ARE seems to act primarily as the most distal subapical station relaying apical cargo from the CE to its (apical) destination, rather than simply recycling it. The SAC of hepatocytes also contains functionally and presumably compositionally distinct proximal and distal subcompartments, and the integrity of the latter is, like the ARE, dependent on microtubules. Although these subapical structures are defined differently in various epithelial cells, we propose that they share similar principal functional and molecular features, and we use SAC and CE interchangeably hereafter.

There is clearly a common, recycling endosomal compartment where apical and basolateral endocytic pathways merge in epithelial cells. However, the requirement for the ARE as a subcompartment along the transcytotic trafficking route to the apical membrane has been questioned on the basis of kinetic modeling of Tf transport to and from the apical and basolateral membranes via the CE in MDCK cells (Sheff et al., 2002). As described above, biochemical evidence supports the existence of a functionally distinct subcompartment such as the ARE, to which Rab11 predominantly localizes. In MDCK cells that overexpress Rab11 mutants, the trafficking of the complex formed by IgA and the polymeric IgA receptor (pIgR) to the apical membrane is disrupted. However, sorting and recycling of Tf continues (Wang, E. et al., 2000). The question arises therefore as to whether apical recycling of Tf, as observed under the experimental conditions (Sheff et al., 2002), requires transport via the ARE or, alternatively, enters a direct pathway from the CE to the apical surface, which does not involve the ARE. Interestingly, in polarized HepG2 cells, GlcCer recycles similarly between the SAC and the apical membrane along a pathway that is not affected by inhibitors that block apically directed transport of SM (upon signaling) and pIgR, which exit the SAC by its ARE-reminiscent, microtubule-sensitive distal part (van IJzendoorn and Hoekstra, 1998; van IJzendoorn and Hoekstra, 1999). Therefore, the possibility cannot be excluded

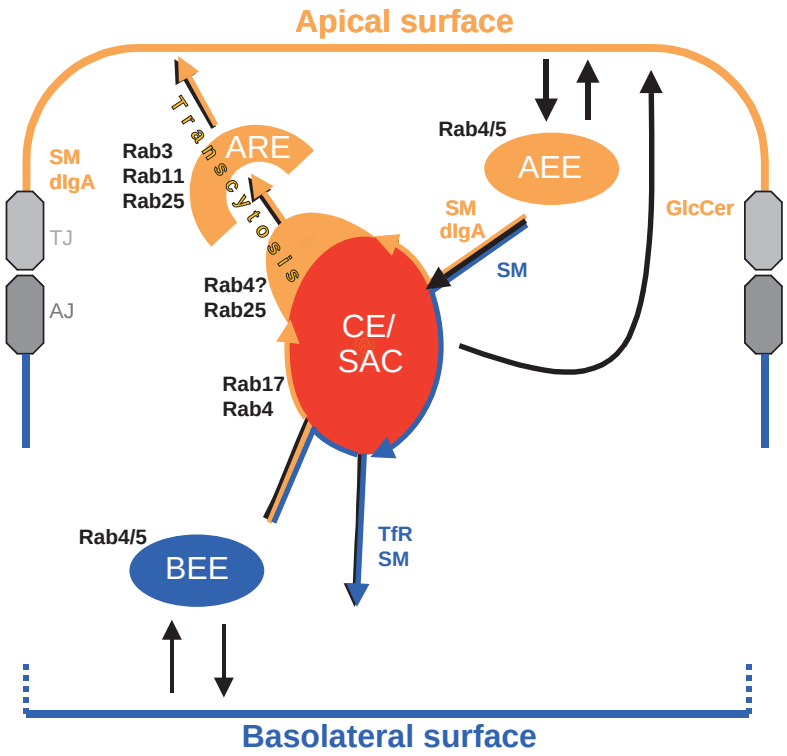

Fig. 3. Organization of the endosomal recycling system in polarized epithelial cells. The SAC/CE is accessible both to apical and basolateral early-endosome-derived transport pathways. Its most distal part is defined as the ARE and is considered an integral subcompartment whose stability is dependent on microtubules. This subcompartment is involved in relaying molecules to the apical surface as the final step in the overall basolateral-to-apical transcytotic pathway. For further details, see text. Colored arrows depict apical or basolateral transport routes. AEE, apical early endosome; AJ, adherens junctions; ARE, apical recycling endosome; BEE, basolateral early endosome; CE, common endosome; dIgA, dimeric immunoglobulin A/pIg-receptor complex; GlcCer, glucosylceramide; SAC, subapical compartment; SM, sphingomyelin; TfR, transferrin receptor; TJ, tight junctions.

that direct recycling occurs to the apical membrane that bypasses the ARE. Obviously, further work will be needed to define better the multiple transport pathways that connect the SAC/CE with the apical and basolateral membranes.

Trafficking to and from the SAC

The SAC receives cargo (Fig. 1), presorted in early sorting endosomes, from both the basolateral (Fig. 1, step 3) and apical surface (step 12) but also connects with lysosomes (step 7) by a prelysosomal compartment. Endolyn-78, a lysosomal-resident glycoprotein internalized from the basolateral surface in the hepatocyte-derived cell line WIF-B (Ihrke et al., 1998) or from the apical surface (i.e. the membrane domain facing the bile canaliculus) in rat hepatocytes in situ (Rahner et al., 2000), accumulates in the SAC prior to reaching a shared $Rab7^+$ and cathepsin-D^+ prelysosomal compartment and, eventually, lysosomes. This is also true for other in situ-internalized apical-resident proteins, including dipeptidylpeptidase IV (DPPIV) and aminopeptidase N (APN). Because material recycling to either domain, as well as transcytotic traffic, also traverses the compartment, the SAC must have a crucial function in the maintenance of membrane polarity and hence harbor sophisticated sorting machinery.

Studies of the transcytosis of the pIgR-dIgA complex, which crosses the CE on its route from the basolateral to the apical surface, and of the trafficking of the Tf-TfR complex, have been imperative in defining the properties of the CE (Rojas and Apodaca, 2002; Mostov et al., 2000; Mostov et al., 2003). Basolaterally internalized Tf-TfR partly recycles through the BEEs (Fig. 1, step 2), but a considerable fraction reaches the CE (Fig. 1, step 3). Once there, the Tf is directly recycled (Fig. 1, step 4; see also Fig. 2). By contrast, the pIgR-dIgA complex moves to the distal, microtubule-sensitive part (the ARE) prior to apical delivery (step 9) (Brown et al., 2000). Interestingly, Tf gains access to the distal $Rab11a^+$ and $Rab25^+$ subcompartment upon mis-sorting in MDCK cells (Wang et al., 2001) treated with brefeldin A, which blocks recycling.

Newly synthesized apical-resident proteins that are targeted to the apical surface by transcytotic transport through the basolateral surface (Fig. 1, step 13) (Ihrke et al., 1998; Aït Slimane and Hoekstra, 2002; Lipardi et al., 1999) (see below) pass through the SAC (Fig. 1, steps 1 and 3). Similarly, C_6NBD-SM and C_6NBD-GlcCer localized in the apical membrane enter the SAC following endocytosis (steps 10 and 12), extensively colocalizing with the pIgR-dIgA complex, prior to their recycling (C_6NBD-GlcCer) or transport (C_6NBD-SM) to the apical (step 8) or basolateral (step 4) surface, respectively (van IJzendoorn and Hoekstra, 1998). The SAC thus occupies a central position in both the basolateral and apical endocytic trafficking pathways. Regulators of these pathways include phosphoinositide 3-kinase (PI 3-kinase) (Foster et al., 2003), specifically the class III type Vps34p (Tuma et al., 2001). When phosphatidylinositol 3-phosphate formation is inhibited by abrogation of PI 3-kinase activity, the basolaterally enriched Rab5 effector EEA1 (Wilson et al., 2000) leaves the BEE, which impedes BEE-to-SAC transport (Fig. 1, step 3), whereas its association with the AEE remains unaffected (Tuma et al., 2001). There are thus differences in the mechanisms that regulate apical and basolateral endocytosis. These might include distinct modes of operation of apical versus basolateral microdomain-localized Rab5/Rab4 (see above), with Rab4 rather than Rab5 being thought to be involved in vesicle assembly and transport.

As well as being central to endocytic trafficking in the context of membrane polarity, does the SAC also contribute to sorting in the biosynthetic pathway (i.e. by being connected to the Golgi; Fig. 1, step 15)? Following arrival in the trans-Golgi network (TGN), proteins are selectively sorted and packaged into distinct carrier vesicles, destined for the apical (step 14) or basolateral (step 13) surface. Direct targeting from the TGN to either surface would be efficient, but not all apical proteins follow such a direct route. Although direct transport predominates in MDCK cells, apical proteins can also reach the apical surface by transcytosis from the basolateral

membrane through the SAC, as observed in Caco2 cells, hepatocytes and FRT cells. Single-span membrane proteins, including glycosylphosphatidylinositol (GPI)-anchored proteins, travel in liver cells along this indirect pathway (Bastaki et al., 2002; Aït Slimane et al., 2003; De Marco et al., 2002), whereas multispan proteins, such as the ABC transporters MDR1, MDR2, Mrp2 and the sister of P-glycoprotein (SPGP), as well as the copper transporter ATP7B, travel directly from the TGN to the apical membrane (Soroka et al., 1999; Kipp et al., 2001; Aït Slimane et al., 2003). Interestingly, in situ immunofluorescence has revealed that one of these proteins (SPGP) localizes to subcanalicular endosomal vesicles (Gerloff et al., 1998) at the apical poles of the cells and travels in a microtubule-dependent manner to the apical, bile canalicular membrane through an endosomal compartment that is enriched in Rab11 (Kipp and Arias, 2000), and is distally positioned in the traffic route through the SAC (Somsel Rodman and Wandinger-Ness, 2000). Therefore, although their origins differ (basolateral membrane versus TGN), the pIgR-dIgA complex and SPGP might have very similar exit pathways from the SAC. Although MDR1 and MDR2 do not seem to travel along the same pathway to the apical membrane in liver cells (Kipp et al., 2001), trafficking of these proteins is sensitive to the cAMP signaling pathway (see below) that activates SAC-derived transport to the apical, bile canalicular membrane in HepG2 cells (Fig. 1, step 9) (van IJzendoorn and Hoekstra, 2000a; van IJzendoorn and Hoekstra, 2000b). However, whether, and if so how much, these proteins cycle between the apical membrane and the SAC remains to be determined.

Machinery in SAC-mediated trafficking

The distinct trafficking pathways through the SAC, together with observable morphological heterogeneity, indicate that it has different (sub-)compartments that might contain distinct lateral microdomains, and these might harbor distinct machineries. Very little is known either of the molecular machinery that governs transcytotic and biosynthetic trafficking, or the apical and basolateral recycling through the CE/SAC. Nevertheless, some aspects of these mechanisms are becoming apparent.

Rab proteins

Distinct Rab proteins become specifically expressed in the course of development of cell polarity, and some of these are closely associated with the SAC (Figs 2, 3). In fact, this is one way (van IJzendoorn and Hoekstra, 1999; van IJzendoorn et al., 2003) in which the SAC acquires properties that distinguish it from the recycling compartment in nonpolarized cells. These Rab proteins often display distinct (subcompartmental) localizations – as do those on early and recycling endosomes in nonpolarized cells – which might be instrumental in the regulation of distinct transport steps. It is also possible that epithelial-specific coat adaptors cooperate with Rab effectors to mediate the biogenesis/budding of different types of transport vesicles.

In MDCK cells, Rab4 localizes to the CE, and its overexpression abrogates recycling of TfR to the basolateral membrane, causing it to redistribute to the apical surface. This suggests that Rab4 has a role in regulating TfR recycling (Mohrmann et al., 2002). Interestingly, actin perturbation causes a very similar mis-sorting (Durrbach et al., 2000; Sheff et al., 2002) (see below), which indicates a potential link between Rab-controlled and cytoskeleton-dependent transport. Rab3 and Rab17 family members are specifically expressed in polarized epithelial cells (Lutcke et al., 1993; Zacchi et al., 1998; van IJzendoorn et al., 2002). Rab3b regulates transcytotic trafficking of pIgR from the ARE. Similarly, Rab3d localizes near the apical plasma membrane in liver cells and colocalizes with the pIgR in transcytotic carrier vesicles, which suggests it is involved in the regulation of a distal step in transcytosis in these cells (Larkin et al., 2000).

Certain Rab proteins (Rab11, Rab17 and Rab25) localize to specific regions of the SAC (Goldenring et al., 1996; Hansen et al., 1999; Casanova et al., 1999; Rahner et al., 2000; van IJzendoorn et al., 2003) or to apical tubules – tubular extensions in the filament-rich region of the apical most part of the cytoplasm [Rab18 and Rab20 (Zerial and McBride 2001; Somsel Rodman and Wandinger-Ness, 2000)]. Overexpresion of dominant-negative Rab17 in polarized Eph4 cells reroutes TfR trafficking from the SAC to the apical, rather than the basolateral domain (Zacchi et al., 1998). In MDCK cells, overexpression of Rab17 inhibits pIgR transcytotic transport and, as in Eph4 cells, the Rab protein colocalizes with apically and basolaterally endocytosed pIgR-dIgA (Hunziker and Peters, 1998). This might mean that TfR and pIgR arriving at the CE follow different pathways to the apical membrane that are differentially regulated by Rab17. This would be analogous to the regulation of apical but not basolateral endocytic trafficking by Rab5, whose effector Vps34 is present at the apical but not the basolateral domain (Tuma et al., 2001). In this scenario, an important feature of the subcompartmentalization of the SAC could be that distinct activities of the same Rab are spatially separated and regulated by different effectors. Apically endocytosed pIgR-dIgA colocalizes with Rab17 in the CE, rather than the ARE, which is consistent with the notion that the Rab17 compartment is insensitive to microtubule perturbation (Zacchi et al., 1998), which is in contrast to the ARE. Similarly, also in liver cells, apically endocytosed molecules are initially delivered to the microtubule-resistant part of the SAC (van IJzendoorn and Hoekstra, 1998; Rahner et al., 2000).

The exact role of Rab11 is unclear. It is associated with the ARE in MDCK cells (Casanova et al., 1999; Wang et al., 2001) and with the microtubule-sensitive distal part (Fig. 3) of the SAC in HepG2 cells (S.C.D.v.IJ. and D.H., unpublished) (Fig. 2). Indeed, Rab11 has been proposed to play a role in microtubule organization and the subsequent spatial organization of membrane compartments and plasma membrane recycling (Dollar et al., 2002). In nonpolarized cells, Rab11 controls the recycling of TfR, possibly by regulating exit from early sorting endosomes (Ren et al., 1998) and docking at the recycling endosome (Zerial and McBride, 2001). In *Drosophila* epithelia, trafficking through Rab11-containing recycling endosomes is essential for remobilizing membrane pools, which are partly derived from the apical surface, in order to ensure lateral membrane growth (Pelissier et al., 2003). Exit from the subapical Rab11-containing compartment, which probably represents the ARE, is regulated by the GTPase shibire/dynamin (Pelissier et al., 2003).

Furthermore, the expression of a dominant-negative Rab11 mutant in polarized gastric parietal cells inhibits the recruitment of the H^+K^+ ATPase from subapical vesicles to the apical surface (Duman et al., 1999). Rab11 might similarly control the exit step from the SAC to the apical membrane in other epithelial cells, since a dominant-negative Rab11 mutant also disrupts pIgR-dIgA transcytosis in MDCK cells, without affecting TfR recycling (Wang, X. et al., 2000).

The underlying mechanism possibly relies on recruitment of the Rab11 effector Rip11 (Meyers and Prekeris, 2002), which is a member of a larger group of Rab11-interacting proteins (Rab11-FIPs) (Meyers and Prekeris, 2002), and/or the actin-dependent motor protein myosin Vb (Lapierre et al., 2001; Hales et al., 2001). Whether Rab11 is involved in actin remodeling and recruitment, possibly for apical membrane expansion (Pelissier et al., 2003), is an interesting possibility that would parallel mechanisms in cytokinesis (Riggs et al., 2003). Finally, like Rab11, Rab25 appears to be involved in regulating distal steps in SAC-to-apical-membrane transport, because its overexpression slows the rate of IgA transcytosis. However, its mechanism of action might be different from that of Rab11 as expression of dominant-negative Rab25, in contrast to dominant-negative Rab11, does not alter transcytosis (Casanova et al., 1999).

cAMP/PKA signaling

In polarized HepG2 cells, C_6NBD-SM and C_6NBD-GlcCer normally localize to distinct domains of the SAC (van IJzendoorn and Hoekstra, 2000b; Maier and Hoekstra, 2003). SM is transported to the basolateral membrane, whereas GlcCer recycles between the SAC and apical membrane, along a pathway different from that taken by pIgR-dIgA, when traveling through SAC to the apical membrane (Fig. 3). Acting through protein kinase A (PKA), cAMP induces polarity development, which is reflected by an increase in the number of cells displaying a polarized phenotype, and an enlargement of the apical surface area (‘hyperpolarization’) of cells already polarized. Specifically, this treatment activates a common calmodulin-dependent SAC-to-apical membrane exit pathway (marked by pIgR-dIgA) that SM now follows. By contrast, the SAC-to-apical membrane pathway marked by GlcCer transport and recycling appears unaffected (Zegers and Hoekstra, 1997; van IJzendoorn and Hoekstra, 2000b). cAMP also stimulates apical transport of Mrp2 in hepatocyte couplets (Soroka et al., 1999) and the apical recycling of V-ATPase (Pastor-Soler et al., 2003), a protein that typically resides in subapical, cup-shaped vesicles (Hermo et al., 2000). Hyperpolarization is similarly apparent under these circumstances (Soroka et al., 1999), which suggests that the pIgR-dIgA-marked pathway from the SAC to the apical surface characterized in HepG2 cells is also activated in hepatocytes. Hence, the SAC appears to contain a PKA sensor capable of reversing SM-marked trafficking, which seems to be physiologically relevant because transient activation of intrinsic PKA occurs at the early stages of polarity development in HepG2 cells (van IJzendoorn and Hoekstra, 2000a).

Microtubule disruption inhibits PKA-promoted (hyper)polarization (Zegers et al., 1998), as well as transcytosis of apical-resident proteins (Tuma et al., 2002). In contrast, recycling of C_6NBD-GlcCer from the SAC to the apical membrane, which is unaffected by PKA activation, also appears independent of microtubules and proceeds normally. This suggests that a recycling pathway that does not require transport through the distal microtubule-sensitive part of the SAC operates between the apical surface and the SAC. These observations are in line with the notion that disruption of trafficking by the ARE in MDCK cells (as reflected by the flow of pIgR-dIgA) does not interfere with sorting and recycling of Tf-TfR complexes (Wang, X. et al., 2000) (cf. Rojas and Apodaca, 2002). Most importantly, in light of the enlargement of the apical membrane area upon PKA activation, these data might thus indicate that the microtubule-dependent exit pathway from the SAC is involved in de novo biogenesis of the apical membrane domain. The regulation of traffic through this pathway also involves Rab11a and Rab3b (Prekeris et al., 2000; van IJzendoorn et al., 2002) (S.C.D.v.IJ. and D.H., unpublished), which localize to distal parts of the SAC and CE (thus implicating the ARE). A similar signal-regulated pathway is responsible for apical transport of the cystic fibrosis transmembrane regulator (CFTR) channel protein (Ameen et al., 1999) and the aquaporin-2 water channel protein (Fushimi et al., 1997) in intestinal epithelia and polarized LLC-PK1 renal cells, respectively.

PKA-regulated trafficking from the SAC is a target for signaling cascades elicited by cytokines belonging to the interleukin (IL)-6 family. Oncostatin M (OSM), a member of this family, is an important regulator of liver maturation, triggering formation of intercellular E-cadherin-based and catenin-based adherens junctions between fetal hepatocytes, which are crucial steps in the differentiation of liver tissue (Matsui et al., 2002). In cell culture, these cytokines promote development of cell polarity by stimulating the biogenesis of the apical domain and binding to receptors at the basolateral surface (van der Wouden et al., 2002). gp130, the signal-transducing unit of the OSM/IL-6 receptor is recruited into sphingolipid/cholesterol-enriched plasma membrane domains, which elicits cross-talk through an as-yet-unknown mechanism with cAMP signaling, and hence implicates PKA activation in IL-6-dependent polarity development (van der Wouden et al., 2002). These data thus provide a clue to the molecular mechanism that couples the biogenesis of the apical membrane domain to the regulation of intracellular transport exiting the SAC in response to an extracellular, basolaterally localized stimulus. The molecular mechanisms remain to be determined but could be similar to those operating in the regulated redistribution of specific integrins to the leading edge of migrating cells in response to signals that act on a Rab11a-controled subset of recycling endosomes (Powelka et al., 2004).

Lipid microdomains

Liquid-ordered sphingolipid/cholesterol-enriched microdomains (lipid rafts) have been proposed as targeting devices in the direct transport of apical proteins from the TGN to the apical membrane (Ikonen and Simons, 1998; Ikonen, 2001; Hoekstra et al., 2003). The GPI membrane anchors, or ecto- and transmembrane domains of these proteins, are thought to function as the sorting signals that target them to the appropriate location (Rodriguez-Boulan and Gonzalez, 1999; Gu et al., 2001; Nelson and Yeaman, 2001). The CE in MDCK cells is rich in raft components, such as sphingomyelin and cholesterol, and the raft-associated proteins caveolin and flotilin (Gagescu et

al., 2000). In fact, distinct sphingolipid domains exist in the SAC (van IJzendoorn and Hoekstra, 2000b), and similar domains have also been identified on Rab11-containing recycling endosomes (Sharma et al., 2003). Recently, Nyasae et al. showed that the delivery of apical-membrane-destined proteins from BEEs to the SAC requires sphingolipids and cholesterol (Nyasae et al., 2003). In HepG2 cells, these proteins appear to localize to detergent-insoluble (Triton X-100) microdomains while transcytosing from the basolateral to the apical membrane domain (Aït Slimane et al., 2003).

MAL2, a member of the MAL family of raft-associated proteins, has recently been identified in HepG2 and Caco2 cells (De Marco et al., 2002). Colocalization studies suggested that MAL2 localizes to the SAC and resides in Triton X-100-resistant microdomains, which is thought to indicate raft localization. In MDCK cells, MAL regulates apical targeting from the TGN to the apical membrane (Puertollano et al., 2001; Martin-Belmonte et al., 2001), and its depletion causes accumulation of apical cargo in the Golgi. MAL2 might have a similar role in apical trafficking from the SAC, since its downregulation impedes transcytosis of both pIgR and the GPI-anchored protein CD59. However, since the proteins largely accumulated in large perinuclear endosomes in these studies (De Marco et al., 2002), and the pIgR does not seem to localize to rafts (Sarnataro et al., 2000; Rojas and Apodaca, 2002), the role of MAL2 in the SAC remains to be determined. It is possible that regulatory proteins such as MAL require rafts as signaling platforms, but not directly for sorting of cargo.

The role of detergent-resistant microdomains in apical trafficking is thus unclear. Single-span apical membrane proteins in liver cells travel by the indirect transcytotic pathway (Ihrke et al., 1998; Aït Slimane et al., 2003) – from the Golgi to the basolateral domain and onward to the apical surface as part of Triton X-100-resistant microdomains (Aït Slimane et al., 2003). This indicates that raft-mediated trafficking operates in both the apical and basolateral pathways. Similarly, polytopic proteins, trafficking directly from the TGN to the apical membrane (Kipp and Arias, 2000; Aït Slimane et al., 2003) enter lubrol-insoluble, but Triton X-100-soluble domains in the Golgi (Aït Slimane et al., 2003). However, one should be cautious about interpreting such results (for a critical discussion, see Chamberlain, 2004). Nevertheless, in this case, the detergent-dependent differences in (in)solubility, lubrol being a weaker detergent than Triton X-100, could reflect the recruitment of different types of protein to distinct membrane microdomains, because one type of raft travels to the basolateral surface and the other travels to the apical surface. Sorting of the lubrol-insoluble domains depends on a cholesterol-sensitive factor because removal of cholesterol eliminates apical sorting but not association of the proteins with the rafts (Aït Slimane et al., 2003). These data indicate that raft recruitment is not sufficient for polarized protein targeting. Nevertheless, it seems to play some role and biochemical characterization of membrane microdomains, including their functional reconstitution, will be imperative if we are to appreciate further their functions in SAC-dependent trafficking.

The cytoskeleton and polarized trafficking

Actin

Actin and actin-binding motor proteins play an important, but poorly understood, role in trafficking to and from the SAC, and possibly in the biogenesis of the SAC. Actin depolarization induced by latrunculin treatment or overexpression of the small GTPase RhoA (Leung et al., 2000) decreases TfR recycling from the SAC to the basolateral membrane in Caco2 cells (Durrbach et al., 2000) and MDCK cells (Sheff et al., 2002), causing mis-sorting to the apical surface. Simultaneously, recycling from the BEE appears compromised (Sheff et al., 2002), and the pool of membrane-bound particles constituting the SAC as observed in control cells is reduced. This could signify an actin-related perturbation in the merging of AEE- and BEE-derived transport vesicles, which might be instrumental in the biogenesis of the SAC. Indeed, under these conditions, basolaterally derived sorting endosomes and transport vesicles fuse directly with the apical surface, thus explaining mis-sorting of basolateral receptors (Durrbach et al., 2000; Sheff et al., 2002), and emphasizing the importance of the SAC as a transcytotic sorting center.

Myosin I and its relative brush border myosin I (BBMI) function in Tf recycling (Apodaca, 2001) and in recycling and transcytosis of the apical-resident protein DPPIV (Durrbach et al., 2000), respectively. Furthermore, expression of a dominant-negative myosin Vb mutant in polarized MDCK cells stably expressing pIgR causes accumulation of basolaterally endocytosed dIgA-pIgR in the ARE. This suggests that actin dynamics play a role in regulating exit from the ARE (Lapierre et al., 2001). Thus, the integrity of actin filaments is imperative for SAC-connected membrane trafficking; whether actin also regulates trafficking within or between SAC subcompartments remains to be determined.

In this context, it is important to comment on the endocytic capacity and dynamics of the apical surface in connection with the SAC. It has been proposed that actin-based scaffolds can actively prevent certain proteins from becoming internalized by endocytosis (Yeaman et al., 1999). In fact, Tuma et al. suggest that this is the case for apical-resident proteins such as APN, 5′NT and pIgR in WIF-B cells until their removal is specifically triggered by a signal for lysosomal delivery (Tuma et al., 2002). Remarkably, actin depolymerization triggers basolateral targeting of these proteins, which is reminiscent of the opposite scenario in which basolateral proteins are mistargeted to the apical membrane under similar conditions (Durrbach et al., 2000; Sheff et al., 2002). However, the extent to which local recycling of apical-resident proteins occurs requires further investigation, particularly since Rahner et al. have reported apical endocytosis of these proteins by liver cells in situ, showing, in contrast to the data from WIF-B cells (Tuma et al., 1999), that the SAC acts as an intermediate compartment that also sorts apical-resident proteins for lysosomal degradation (cf. Figs 1 and 2) (Rahner et al., 2000). The dynamic interaction between the apical surface and the SAC is also highlighted by the notion that apically localized sphingolipids are readily endocytosed and reach the SAC for recycling in the case of GlcCer or further transport to the basolateral membrane in the case of SM (van IJzendoorn and Hoekstra, 2000b). Moreover, uncleaved pIgR-dIgA recycles from the apical membrane via AEEs to the CE, before transferring to the apical membrane via the microtubule-sensitive Rab11-containing ARE (Wang, X. et al., 2000).

Microtubules

The apical and basolateral membranes are distinctly oriented with respect to microtubule polarity: microtubule plus ends face the basolateral membrane, whereas their minus ends face the apical membrane (Allan et al., 2002). This creates the potential for selectivity through use of motors that have different directionality. These presumably bind to apical or basolateral transport vesicles and are themselves sorted. As discussed above, the SAC contains a microtubule-depolymerization-sensitive subcompartment, and microtubules are thus crucial to its structural and hence functional organization. Moreover, apically endocytosed sphingolipid analogs accumulate in apical endocytic vesicles and do not reach the SAC upon disruption of the microtubules. Thus, inbound and outbound trafficking rely heavily on an intact microtubule network, and microtubule-based motors probably play an important role in SAC-dependent trafficking. Transcytotic vesicles enriched in pIgR, DPPIV, Pgp and Mrp2 from rat liver are highly enriched in the minus-end-directed microtubule motor dynein (Soroka et al., 1999), and this motor is also thought to drive pIgR-dIgA transport from the BEEs to the CE (Rojas and Apodaca, 2002). Although this vesicle fraction is heterogeneous in nature, part of it might be related to the distal, microtubule-sensitive part of the SAC. Following biosynthesis, pIgR and DPPIV reach the apical membrane by transcytosis via the SAC and colocalize in these vesicles with MDR1, which travels by the direct route (see above). It remains to be determined whether this implies that MDR1 actually recycles via the SAC and follows a dynein-driven pIgR/dIgA-marked pathway to the apical membrane, but this would be consistent with its entry into the cAMP-sensitive apical pathway following apical endocytosis (Kipp et al., 2001).

The kinesin-related motor Unc 104 (KIF1A) clusters in lipid rafts, which might trigger its activity, and binds to phosphatidylinositol (4,5)-bisphosphate through its pleckstrin homology (PH) domain (Klopfenstein et al., 2002). There might therefore be another level at which cytoskeletal motors operate in SAC-dependent trafficking: as part of raft-associated sorting devices.

Perspective

There is good evidence to suggest that the SAC/CE functions as a polarized recycling system, but its physiological role remains unclear. For instance, when transport to the SAC/CE is perturbed, recycling basolateral plasma membrane macromolecules can instead be delivered from basolateral early endosomes to the apical surface. However, efficient recycling from basolateral and, presumably, apical early endosomes also occurs without a need for distal recycling endosomal elements such as the SAC/CE. This leaves the intriguing question as to the relevance of such an elaborate and molecularly sophisticated recycling and sorting system. An attractive hypothesis is that the SAC/CE provides an intracellular membrane pool that can be readily recruited for the assembly of specialized plasma membrane domains, like a migrating front or a lateral or apical surface domain, and is equipped with a specific subset of proteins and lipids. Indeed, several lines of evidence have pointed to the involvement of Rab11, a typical recycling endosomal GTPase, in these processes. Moreover, polarized transport from the recycling system has been shown to be a target for extracellular signals, including those that regulate apical plasma membrane biogenesis and cell migration, as well as those that control the appearance of transporter and channel proteins at the apical surface.

Current work on cell (membrane) polarity has largely focused on its development and maintenance, as well as the basic characteristics of endocytic and transcytotic pathways; the result is that compartments through which cargo travels en route to either membrane domain are now defined to some extent. Insight into the initiation of polarity development is also gradually emerging (Nelson, 2003; Mostov et al., 2003). The next challenge is to identify the underlying molecular mechanisms, which will make the isolation of the SAC and its detailed biochemical characterization inevitable. Progress in this area will also provide insight into how the SAC acquires its characteristic properties in fully polarized epithelial cells, which will deepen our understanding of how membrane polarity is established.

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