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COMMENTARY

Establishing and maintaining epithelial cell polarity

Roles of protein sorting, delivery and retention

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Introduction

Polarized epithelial cells are distinguished from many other cell types by the presence of distinct plasma membrane domains, termed apical and basal-lateral, which face different biological compartments separated by the epithelium (Rodriguez-Boulan and Nelson, 1989). An important problem is to understand the mechanisms involved in the development of the structural and functional organization of these membrane domains. It has been generally thought that membrane domains are generated and maintained by sorting membrane proteins in the *trans*-Golgi network (TGN) into different vesicle populations, which are then delivered directly to the appropriate membrane domain (Simons and Wandinger-Ness, 1990). Here, we summarize recent studies on potential signals involved in the sorting of proteins in the TGN, and discuss them in the broader context of how cells generate cell surface polarity during development.

We will consider three sequential steps that are part of the protein sorting machinery in these cells, and which *together* appear to be involved in generating and maintaining polarized distributions of proteins on the epithelial cell surface: (1) sorting of apical and basal-lateral membrane proteins in the TGN into different populations of transport vesicles; (2) transport to, and capture and docking of the transport vesicle at the appropriate cell surface domain; (3) retention of the protein in the membrane domain.

Sorting signals for apical and basal-lateral membrane proteins in MDCK cells

Recent studies using the polarized epithelial cell line MDCK (Madin-Darby canine kidney) have begun to yield compelling evidence for the existence of different sorting signals that mediate protein delivery to either the apical or the basal-lateral membrane.

One likely sorting signal for the direct delivery of proteins to the apical membrane involves anchorage of proteins to the lipid bilayer through glycosylphosphatidylinositol (GPI) (Lisanti et al. 1988; Lisanti et al. 1989; Brown et al. 1989). Since glycosphingolipids are also sorted in the TGN to the apical membrane (van Meer et al. 1987), it has been proposed that sorting of GPI-anchored proteins in the TGN may be through their incorporation with glycosphingolipids into glycosphingolipid rafts, or clusters (Simons and van Meer, 1988). Of course, not all proteins that are sorted to the apical membrane contain a GPI-anchor. Studies of non-GPI-linked apical proteins indicates that sorting information, if present, is located in the ectodomain of the protein (Roth et al. 1987; see below). It has been proposed that an ectodomain sorting signal could be recognized by a putative apical sorting receptor, which, in turn, interacts with glycosphingolipids (Simons and Wandinger-Ness, 1990).

Formation of glycosphingolipid clusters in the TGN could exclude other (basal-lateral?) membrane proteins that have little or no affinity for glycosphingolipids (Lisanti and Rodriguez-Boulan, 1990). Proteins excluded from the glycosphingolipid rafts could be incorporated into a separate vesicle population that is then delivered, by default, to the basal-lateral membrane (Simons and Wandinger-Ness, 1990). Although the implication is that this "default pathway" does not require a sorting signal, the exclusion of these proteins from glycosphingolipid clusters and vesicles destined for the apical membrane can be considered a form of signal-mediated sorting, albeit a negative one.

However, recent evidence strongly indicates the existence of a positive sorting signal for basal-lateral membrane proteins in these cells. Four independent approaches have been used. An apically sorted protein has been converted to one that is delivered to the basal-lateral membrane (Brewer and Roth, 1991; Le Bivic et al. 1991), or minimum sequences specifying basal-lateral delivery of proteins have been defined by analysis of the sorting of truncated and chimeric proteins (Hunziker et al. 1991a; Casanova et al. 1991).

The path towards the identification of a putative basal-lateral sorting signal was initiated in studies on "non-polarized" fibroblasts by Lazarovits and Roth

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(1988), who showed that conversion of a cysteine to a tyrosine at position 543 in the cytoplasmic domain of the viral protein hemagglutinin (HA-Y_{543}) caused the mutated protein to be efficiently internalized through coated vesicles at the cell surface; by comparison, internalization of wild-type HA was very poor. Lazarovits and Roth (1988) proposed the hypothesis that tyrosine at position 543 caused a conformational change in the secondary structure of the protein to form a bend in the polypeptide chain that was recognized as a signal for internalization via clathrin-coated vesicles. Recently, this structure has been shown to be a tight β-turn (Bansal and Gierasch, 1991; Eberle et al. 1991).

In polarized MDCK cells wild-type HA is sorted from basal-lateral proteins in the TGN, perhaps by association with glycosphingolipids (Skibbens et al. 1989), and is then delivered directly to the apical membrane (Matlin and Simons, 1983; Rindler et al. 1984). Significantly, Brewer and Roth (1991) showed that HA-Y_{543} was efficiently sorted to the basal-lateral membrane rather than being delivered to the apical membrane. Thus, the signal for endocytosis in HA not only conferred delivery to the basal-lateral membrane, but it was also dominant to the inherent sorting signal in the protein for delivery to the apical membrane.

At the same time, Le Bivic et al. (1991) were analyzing the delivery of nerve growth factor receptor (NGFR) to the cell surface in MDCK cells. They found that wild-type NGFR, like HA, was delivered to the apical membrane, and that NGFR was internalized from the apical membrane domain at a very low rate (6%). However, an internal deletion of 58 amino acids in the cytoplasmic domain of NGFR resulted in a mutant receptor that was efficiently delivered to the basal-lateral membrane. In addition, the truncated NGFR exhibited a >100-fold increase in the amount of ligand-induced endocytosis compared to the wild-type receptor. Further analysis of this mutation revealed that a tyrosine residue (Y_{308}) was moved into close proximity with the transmembrane domain.

Together, the studies by Brewer and Roth (1991) and Le Bivic et al. (1991) show that alterations in the cytoplasmic domain of proteins normally sorted to the apical membrane of MDCK cells can result, instead, in delivery of the proteins to the basal-lateral membrane. Thus, at least for these proteins, a basal-lateral sorting signal is dominant to an apical sorting signal, and the signal for basal-lateral sorting correlates strongly with that for cell surface endocytosis.

A different approach to defining a basal-lateral sorting signal has been taken by Hunziker et al. (1991a) and Casanova et al. (1991), who have analyzed the sorting of the Fc and LDL (low density lipoprotein) receptors, and the poly(IgA) receptor, respectively. Analysis of IgG Fc receptor (FcR) sorting in MDCK cells showed that two closely related receptors were sorted to different membrane domains and had different rates of endocytosis at the cell surface via coated vesicles (Hunziker and Mellman, 1990). The receptor carrying an endocytosis signal (FcRII-B2) was delivered to the basal-lateral membrane, whereas the other receptor (FcRII-B1) was delivered equally to both membrane domains (Hunziker and Mellman, 1990). Significantly, deletion of the endocytosis signal of FcRII-B2 resulted in efficient expression of the truncated protein on the apical membrane (Hunziker et al. 1991a).

This result supported the hypothesis that a basal-lateral sorting signal may be similar to the signal for cell surface endocytosis. However, analysis of LDL and poly(IgA) receptors showed that these signals may not necessarily be the same. The LDL receptor has a well-characterized signal for endocytosis that contains a tyrosine or an aromatic amino acid (Davis et al. 1987). Hunziker et al. (1991a) found that mutations in the LDL receptor in which this critical tyrosine was replaced by alanine resulted in a receptor that was delivered efficiently to the basal-lateral membrane, but was poorly internalized at the cell surface.

Casanova et al. (1991) have been studying sorting signals in the delivery of poly(IgA) receptor from the TGN to the basal-lateral membrane. A series of deletions in the cytoplasmic domain of this receptor showed that a 14 amino acid sequence next to the transmembrane domain is sufficient for delivery of the receptor to the basal-lateral membrane. Although this sequence contains a tyrosine residue as the penultimate C terminus residue, the truncated receptor was poorly internalized from the cell surface (Casanova et al. 1991), suggesting that it did not contain a signal for cell surface endocytosis.

Significantly, Casanova et al. (1991) could transfer this putative basal-lateral sorting signal from the poly(IgA) receptor to an apical membrane protein. When the transmembrane domain and the 14 amino acid signal from poly(IgA) receptor were spliced onto the ectodomain of placental alkaline phosphatase (PLAP), the chimeric protein was efficiently delivered to the basal-lateral membrane (Casanova et al. 1991). Note that wild-type PLAP is a GPI-linked protein that is normally delivered to the apical membrane in these cells (Brown et al. 1989). Significantly, fusion of the coding sequences for the ectodomain of PLAP to the transmembrane domain and only two of the 14 amino acids of the cytoplasmic domain of poly(IgA) receptor resulted in a chimeric protein that was still delivered to the apical membrane (Casanova et al. 1991). These results can be interpreted as follows: (1) the signal for sorting to the basal-lateral membrane is transferable to another protein; (2) there are two apical sorting signals in PLAP (the GPI anchor and within the ectodomain); (3) the basal-lateral sorting signal is dominant over the apical signal within the ectodomain of PLAP; and (4) the basal-lateral signal is probably contained in residues 3-14 of the cytoplasmic domain proximal to the transmembrane domain.

Taken together, these recent results strongly suggest that there are positive sorting signals that specify protein delivery to either the basal-lateral membrane, or the apical membrane domain of MDCK cells. In the absence of any sorting signal, proteins may be delivered to both membrane domains. For example, FcRII-B1

was delivered to both cell surfaces (Hunziker et al. 1991a); Na/K-ATPase may also represent another protein that does not possess a strong sorting signal in MDCK cells (Hammerton et al. 1991). These results imply that exclusive delivery to either surface requires some sorting information in the protein. The putative basal-lateral sorting signal has been characterized in four ligand-receptor proteins (NGFR, LDLR, PIgR and FcR). Do non-receptor proteins in the basal-lateral membrane contain a similar signal, or is another type of signal used? Further analysis is needed to clarify the universality of this basal-lateral sorting signal.

Recognition of apical and basal-lateral membrane sorting signals in the TGN

The sorting machinery that recognizes and distinguishes between an apical and basal-lateral membrane sorting signal is not known at present. However, given the nature of the putative sorting signals it seems likely that different classes of proteins are positively sorted by a process of protein clustering in the plane of the lipid bilayer, and the subsequent budding of separate vesicles from the TGN. The clustering of proteins destined to form apical vesicles may be mediated by the aggregation of glycosphingolipids (see above). Adaptor proteins, perhaps similar to the adaptins that are involved in clathrin-mediated coated pit assembly at the cell surface (Pearce and Robinson, 1990), may recognize a basal-lateral signal in the cytoplasmic domain of some proteins and facilitate the clustering of those proteins into a budding vesicle in the TGN. Recently, Hunziker et al. (1991b) have shown that Brefeldin A, a drug that causes the dissociation of adaptin-like proteins from the TGN, specifically inhibits the transcytosis of the poly(IgA) receptor. However, this drug had no effect on sorting of proteins in the TGN, basal-lateral protein targeting or recycling of poly(IgA) receptor to the basal-lateral membrane in MDCK cells. It is possible that different adaptin-like proteins are involved in the sorting and transport of subclasses of proteins destined for distinct membranes. Further analysis of adaptins in polarized epithelial cells may yield important information regarding the mechanism by which different vesicles are delivered to their target membrane.

Sorting signals and establishing epithelial cell surface polarity

It is noteworthy that neither of the candidate signals described thus far for sorting apical or basal-lateral membrane proteins is unique to polarized epithelial cells. Proteins carrying these signals are processed to the cell surface of a wide variety of cell types, including neurons, muscle cells and fibroblasts. These observations raise an interesting question. How can these sorting signals *per se* be responsible for the unique structural and functional organization of membrane domains in polarized epithelial cells if they are also characteristic of non-polarized fibroblast cells? Perhaps non-polarized cells, like their polarized counterparts, sort membrane proteins into separate populations of vesicles in the TGN, but, because there is little or no positional information on the membrane for distinguishing structural or functional differences, they are delivered randomly to the cell surface.

Requirements for positional information to define apical, basal and lateral membrane domains

What is the nature of positional information at the cell surface that defines different membrane domains? Analysis of the conversion of "non-polarized" precursor cells to polarized epithelial cells during early development coincides with the induction of specific cell-cell and cell-substratum contacts (Fleming and Johnson, 1988; Hay, 1990). These extracellular cues could be sufficient to distinguish physically between two cell surface domains: a "bounded" surface in contact with other cells or the substratum, and a "free" surface that is not in contact with other cells or the substratum.

Direct evidence that different extracellular cues play an important role in cell surface and cytoplasmic polarity was shown by analysis of the generation of membrane domains in three-dimensional multicellular cysts of MDCK cells in suspension culture (Wang et al. 1990a; Wang et al. 1990b). Single MDCK cells in suspension culture did not show evidence of cell surface polarity. However, induction of cell-cell contacts resulted in the formation of multicellular aggregates in which marker proteins of the basal-lateral membrane (e.g. E-cadherin, Na/K-ATPase) were restricted in their distributions to the contact sites between cells. Conversely, a marker protein of the apical membrane (gp135) was restricted to the free, unbounded cell surface. Hence, cell-cell contact was both necessary and sufficient to induce the formation of distinct membrane domains in these cell aggregates. Significantly, ZO-1, a marker protein of the tight junction, did not appear to become restricted to its characteristic position at the boundary between the apical and lateral membrane domains until an endogenously secreted extracellular matrix had accumulated in the center of the forming cyst. Thus, cell-substratum contact, at least under these conditions, appears to provide positional information for establishing the apico-basal axis of polarity (Wang et al. 1990a; see also Fleming and Johnson, 1988).

Independent evidence that E-cadherin plays a direct role in inducing the remodelling of membrane protein distributions following cell-cell contact was obtained in fibroblasts transfected with E-cadherin cDNA (McNeill et al. 1990). Under these conditions, the distributions of Na/K-ATPase and E-cadherin became co-restricted to the membrane at cell-cell contacts. This result implies that the ability to recognize and interact with an adjacent cell through E-cadherin contacts provides sufficient information to the cell to restrict another membrane protein to a discrete area (domain) of the cell surface.

Although cell-cell contacts appear to play an important role in establishing polarity of basal-lateral membrane proteins, it is clear from other studies that cell-substratum interactions without cell-cell contact can also act as an important extracellular cue for cell surface polarity. Analysis of single MDCK cells growing on a substratum revealed that a number of membrane proteins are restricted in their distributions to the "free" (apical) cell surface (Vega-Salas et al. 1987; Ojakian and Schwimmer, 1988). The polarity of these proteins may be generated by direct delivery from the TGN, since, under similar conditions, it appears that >70% of all cell surface membrane proteins and >80% of apical proteins are delivered directly from the TGN to the free (apical) membrane (see Wollner et al. 1992). This indicates that, in the absence of cell-cell contact, cells are capable of constitutively sorting proteins into apical and basal-lateral transport vesicles in the TGN.

However, these vesicles must then recognize and interact with the appropriate membrane domain. The cellular apparatus responsible for this second transport and recognition step for basal-lateral vesicles requires >36 hours after the induction of cell-cell contact to become functional (Wollner et al. 1992). This time may be required for the reorganization of the cytoskeleton (Nelson, 1992). In addition, a docking system may be required at the cell surface to capture specific transport vesicles, and subsequently retain cargo proteins in the membrane (see below).

Roles of protein sorting in the TGN and retention in the correct membrane in establishing epithelial cell polarity

Contact between cells appears to be the first signal that leads to the development of a polarized distribution of both apical and basal-lateral membrane proteins in epithelial cells (Vega-Salas et al. 1987; Wang et al. 1990a). By analyzing the development of polarity following the initiation of contact between cells, it is possible to determine what roles protein sorting, vesicular transport and delivery to the cell surface play in establishing distinct membrane domains. Analysis of cadherins and Na/K-ATPase showed different kinetics of development of cell surface polarity (Hammerton et al. 1991; Wollner et al. 1992). Following the induction of cell-cell contacts, different cadherins rapidly became restricted to the forming basal-lateral membrane (<8 h), whereas Na/K-ATPase required a longer period (>36 h). Another study, using monoclonal antibodies that recognized apical and basal-lateral membrane proteins of unknown functions also demonstrated different times for establishing cell-surface polarity of proteins (Vega-Salas et al. 1987).

Significantly, vectorial delivery of newly synthesized proteins from the TGN to the forming basal-lateral membrane do not appear to be required for the development of cell surface polarity of basal-lateral membrane proteins (Hammerton et al. 1991; Wollner et al. 1992). At times when cadherins and Na/K-ATPase had become restricted to the basal-lateral membrane domain (see above), newly synthesized proteins were delivered to both the apical and basal-lateral membranes. Vectorial delivery of cadherins from the TGN to the basal-lateral membrane did not occur until >30 h after cell surface polarity of these proteins had been established. In the case of Na/K-ATPase, newly synthesized protein was always delivered to both cell surface domains (Hammerton et al. 1991). Detailed analysis of the fate of cadherins and Na/K-ATPase upon arrival at the cell surface showed that the proteins had different half-lives at the apical and basal-lateral cell surfaces; 1-2 h in the apical membrane, and 24-36 h in the basal-lateral membrane. This 20- to 30-fold difference in residence times provides an explanation for how cell surface polarity of these proteins is generated in the face of non-polarized delivery of newly synthesized proteins from the TGN to both cell surface domains (Hammerton et al. 1991; Wollner et al. 1992).

What are the mechanism(s) involved in the retention of proteins in the basal-lateral membrane? Previous studies have shown that cadherins and Na/K-ATPase bind to elements of the membrane-associated cytoskeleton (Nelson and Veshnock, 1987a,b; Nelson and Hammerton, 1989; Nelson et al. 1990). It is possible that specific cell-cell contacts, induced through cadherin binding, act as nucleation sites on the (lateral) cell surface for the assembly of the membrane-cytoskeleton. As a consequence of the assembly of the membrane-cytoskeleton, other membrane proteins that are bound to the membrane-cytoskeleton (e.g. Na/K-ATPase) are selectively incorporated into the forming cytoskeletal complex at cell-cell contacts. Since cell-cell contacts are not formed at the apical membrane, there is no signal for membrane-cytoskeleton assembly and, hence, Na/K-ATPase and cadherins are rapidly removed from those regions of the membrane, causing their short residence time at that membrane. Thus, the membrane-associated cytoskeleton may act as a retention system that prevents removal of proteins from the membrane via internalization.

It is not known if other proteins bind to the membrane cytoskeleton or whether other mechanisms are involved in the retention of proteins in the membrane (e.g. interaction with glycosphingolipid rafts, phosphorylation and/or dephosphorylation). For instance, it is interesting to note that inhibition of phosphorylation of the poly(IgA) receptor inhibits transcytosis of the protein from the basal-lateral to the apical membrane domain. The nonphosphorylated receptor is internalized, but recycles back to the basal-lateral membrane resulting in the apparent retention of the receptor in the basal-lateral membrane (Casanova et al. 1990). It is also known that some receptors recycle back to the membrane from which they were internalized (Simons and Fuller, 1985; Rodriguez-Boulan and Nelson, 1989). Preliminary results indicate that one of the cadherin proteins, Dg-1, is phosphorylated 20 times more in the apical membrane than in the basal-lateral membrane (Wollner and Nelson, unpublished results); Dg-1 is rapidly removed from the apical membrane,

but is retained in the basal-lateral membrane (Wollner et al. 1992). It is not known how the phosphorylation state of these proteins correlates with their residence time in the membrane; it is possible that phosphorylation acts as a positive signal for recognition and processing by the endocytosis machinery (see Casanova et al. 1990).

Conclusions and perspectives: defining components of the protein sorting machinery in polarized epithelial cells

We suggest that there are three sequential steps in the sorting process that give rise to a restricted distribution of membrane proteins at the cell surface: (1) sorting of apical and basal-lateral membrane proteins in the TGN into different populations of transport vesicles; (2) capture and docking of the appropriate transport vesicle at the cell surface; and (3) retention of the protein in the appropriate membrane domain.

As summarized above, recent studies have identified candidate signals for sorting of apical and basal-lateral membrane proteins, respectively, in the TGN. However, the machinery involved in distinguishing between these, and perhaps other signals, in the TGN are poorly understood. In vitro reconstitution of vesicle budding and transport from the Golgi complex to the plasma membrane should provide an approach to this problem. The mechanisms involved in transport and docking of transport vesicles with the appropriate membrane domain are also poorly understood. An important recent advance has been the isolation of apical and basal-lateral transport vesicles from polarized MDCK cells (Bennett et al. 1988; Wandinger-Ness et al. 1990; Gravotta et al. 1990). The detailed characterization of these vesicles should provide insight into candidate proteins involved in the sorting, transport and docking processes.

Isolated apical and basal-lateral vesicles have been shown to bind to microtubules in vitro (van der Sluijs et al. 1990), suggesting that microtubules may be important in delivery of vesicles, perhaps via different motor proteins (kinesin, dynein), to either the apical or the basal-lateral membrane. The distribution and polarity of microtubules could provide a suitable spatial framework for the transport component of the sorting process (Nelson, 1992). However, studies of the role of microtubules in vesicle delivery in polarized epithelial cells in vivo require further study (Salas et al. 1986; Rindler et al. 1987; Parczyk et al. 1989; Achler et al. 1989).

The final components of the sorting process are docking and retention of proteins in the appropriate membrane domain. If an endocytosis-like signal proves to be responsible for the sorting of proteins to basal-lateral vesicles, activation of a retention system at the cell surface could be critical in blocking the recognition of that signal by the cell surface endocytosis machinery, which would result in the rapid internalization of the protein and its loss from the cell surface. However, it is also important to consider that some internalized proteins may be recycled to their original (basal-lateral) membrane in a process that would account for their retention at the basal-lateral membrane.

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