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Protein and lipid sorting from the *trans*-Golgi network to the plasma membrane in polarized cells

Elina Ikonen and Kai Simons†*

The targeting of proteins and lipids to the cell surface domains of polarized cells is not a simple bulk flow process but requires sorting into distinct apical and basolateral pathways from the trans-*Golgi network. Here, we describe the sorting determinants in the cargo molecules, the cellular sorting machineries responsible for the hierarchical read-out of the signals, and the mechanisms of cargo delivery. Furthermore, we discuss the implications of these findings for protein–lipid interactions in other cellular machineries.*

Key words: epithelial cell / lipid and protein transport / sorting signal-membrane raft

Introduction

THE ESTABLISHMENT OF asymmetric organization of cell surface domains has been extensively studied using simple epithelial cells, such as Madin–Darby canine kidney (MDCK) cells as models. In these cells, the apical plasma membrane is specialized for functions required at the interface to the external milieu, e.g. the lumen of the renal tubule, and the basolateral cell surface is in contact with the neighbouring cells and the underlying connective tissue.[1] In MDCK cells, this polarized organization is generated by diverting the apically and basolaterally destined cargo into separate transport routes intracellularly, the principal sorting station being in the *trans*-Golgi network (TGN) (for review see ref 2). It is there that separate apical and basolateral carriers form to deliver their contents to the correct cell surface domains. The apical and basolateral domains differ not only in the protein but also in their lipid composition.[3] The external leaflet of the apical plasma membrane is enriched in glycosphingolipids whereas the respective leaflet in the basolateral domain has a high phosphatidylcholine content. Mixing of the membrane constituents in the outer leaflets is prevented by tight junctions.[1]

During the past years, we have gained increasing insight into the rules governing apical and basolateral sorting and into the internal hierarchies that regulate delivery. This review highlights recent advances in the field and illustrates some of the implications these findings bear on other systems.

Two different sorting principles

It now seems clear that neither the apical nor the basolateral route functions as a 'default' pathway, i.e. specific signals are required for the inclusion of cargo both to the apical and the basolateral carrier intermediates. Basolateral sorting signals are confined to the cytosolic tails of membrane proteins. These signals are often tyrosine- or dileucine-based amino acid motifs that may be related to clathrin coated pit endocytic signals.[4] Apical sorting signals are more variable. The most notable difference between the apical and basolateral sorting principles seems to be that apical recognition is based not only on cytosolic protein–protein interactions but also cooperative inter-lipid and protein–lipid affinities as well as carbohydrate recognitions play important roles.[2,5] Fundamental is also the insight that proteins are not strictly either apical or basolateral. This distinction is blurred by the fact that in different epithelial cell types apical proteins can be sorted to the basolateral side and vice versa. The plasticity in sorting is also evident in the variable routes chosen by different proteins in heterologous cells.[6,7] In MDCK cells most apical proteins are sorted directly from the TGN to the apical membrane while in other cell types some

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*From the * Department of Biochemistry, National Public Health Institute, Mannerheimintie 166, 00300 Helsinki Finland and †Cell Biology Programme, European Molecular Biology Laboratory, Meyerhofstrasse 1, 69117 Heidelberg, Germany and Max Planck Institute for Molecular Cell Biology and Genetics, Dresden, Germany*

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or all apical proteins use a basolateral route from the TGN and switch to a transcytotic route basolaterally to reach their apical destination.[8] During cell differentiation proteins may use the indirect transcytotic pathway to begin with and switch to the direct route when the cells mature.[9] The polarity of apical and basolateral proteins may also be regulated by the physiological state of the cell.[10] In response of the kidney to an acid load the polarized distribution of a H^+ATPase and a Cl^-/HCO_3 exchanger changes. This reversal is effected by the polarized secretion of an extracellular matrix protein.[11]

Basolateral sorting

Basolateral proteins not only use tyrosine- or dileucine-based motifs as sorting signals but also other unrelated motifs in their cytosolic tails.[12–15] It has been amazingly difficult to identify the cellular proteins that recognize these sorting signals despite intensive attempts in several laboratories. This may be due to low affinities characterizing the interactions between the cytosolic tail signals and the sorting proteins. Perhaps more than one type of an interaction is involved implying that several proteins have to bind simultaneously to the cargo proteins. Also post-translational modifications might be required for binding between sorting signal and sorting receptor to occur.[16]

A very interesting example of multiple proteins being responsible for the basolateral localization of a protein has recently been unravelled in *C. elegans*.[17,18] A protein complex consisting of LIN-2, LIN-7 and LIN-10 mediates the sorting of the EGF-receptor like protein LET-23. It is LIN-7 that through its PDZ domain binds to the C-terminal TCL-sequence in LET-23. The LIN-2/LIN-7/LIN-10 complex is conserved from worms to humans. An interesting question is whether the LIN-2/-7/-10 complex binds to LET-23 in the TGN and targets the receptor to the basolateral side or whether the binding occurs first after delivery to the plasma membrane, resulting in specific retention of the receptor basolaterally. This latter principle is responsible for the retention of basolateral proteins such as Na^+/K^+-ATPase, the interactions involving fodrin and ankyrin that form part of the cortical cytoskeleton underlying the plasma membrane.[19] It is possible that the assembly of the cytocortex could already be initiated before delivery to the cell surface and be part of the sorting machinery for correct surface delivery.

Apical sorting

Our working model postulates that the basis for apical sorting is provided by lipid interactions, in the form of cholesterol–sphingolipid microdomains forming in the lumenal leaflet of the Golgi membranes.[5] Glycosphingolipids associate into clusters linked together by hydrogen bonds involving the sugar head groups and acyl chains. Furthermore, sphingolipids, especially glycosphingolipids have high melting temperatures due to the saturation of their hydrocarbon chains. Cholesterol is intercalated between the hydrocarbon chains where it can decrease their conformational flexibility and condense the lipid bilayer (for review see ref 20). This lateral packing of sphingolipids and cholesterol results in the formation of dispersed liquid ordered phase domains,[21] rafts, within the otherwise fluid (i.e. liquid disordered phase) TGN bilayer, and functions as a platform for the inclusion of protein cargo destined for delivery to the apical membrane. When the mass fraction of sphingolipids and cholesterol in the membrane further increases, as happens upon mixing of the cargo after delivery to the apical membrane the liquid ordered phase may become continuous. The apical membrane is therefore likely to represent a percolating raft domain where the liquid disordered phase forms isolated clusters.[22]

Raft proteins and lipids can be isolated biochemically as low density Triton X-100 insoluble complexes (DIGs). The DIGs, however, represent substantially larger aggregates than the small rafts dispersed *in vivo* within liquid disordered membranes. Although the actual size of rafts still remains open recent data show that they are considerably small and below the resolution of the microscopical techniques applied so far. Chemical cross-linking in living cells suggests that a glycosylphosphatidyl inositol (GPI)-anchored protein resides in microdomains of at least 15 molecules.[23] Moreover, a fluorescence resonance energy transfer technique estimates the raft diameter to be less than 70 nm.[24]

Apical sorting signals

What do we know about the protein determinants conferring raft association and sorting to the apical transport containers? Proteins that are membrane-anchored either to the exoplasmic or cytosolic phase of the bilayer by saturated acyl chains have affinity for rafts. GPI-anchored proteins are bound to the exoplasmic leaflet by glycosylphosphatidyl inositol with two or more saturated acyl chains[25–27] whereas doubly acylated src family tyrosine kinases bind to the cytosolic side of raft membranes.[26–29] Importantly, clustering of GPI-anchored placental alkaline phosphatase leads to copatching of doubly acylated Fyn suggesting cooperative interactions between the raft bilayer leaflets.[30] Communication across the membrane could be mediated by transmembrane adaptor proteins and involve transbilayer lipid interactions, e.g. interdigitating acyl chains or cholesterol dimers. Also covalent protein–lipid contacts could be involved, an example of an interesting novel modification being the linkage of cholesterol to hedgehog.[31] The structural requirements for transmembrane proteins to associate with rafts have been most thoroughly investigated using the viral protein hemagglutinin (HA) where the membrane spanning segment seems to be critical. The association of HA in detergent-insoluble complexes is sensitive to changes in the sequence spanning the outer membrane leaflet[27] and mutations of residues predicted to lie near the base of the outer leaflet reverse the polarity of HA.[32] The HA protein is also palmitoylated[33] and these hydrocarbon chains may also contribute to raft stabilization.

Another sorting determinant for apical delivery is attributed to *N*-glycans.[34] Not only secretory proteins are sorted apically as a consequence of N-glycosylation but also apical membrane proteins rely on *N*-glycans for apical delivery.[35] Moreover, recent studies imply that O-glycosylation,[36] NeuAcα2-3 glycosylation[37] and chondroitin sulfate (K. Prydz, personal communication) can also carry apical sorting information. Therefore inclusion into apical containers seems to involve a variety of carbohydrate determinants and this in turn implies that there should be a family of apical lectins responsible for apical recognition. The only lectin so far identified is VIP-36 which has homology to leguminous lectins.[38] Another mammalian member of this family is ERGIC-53 which cycles between the ER and the Golgi complex. This lectin recognizes mannose chains and is involved in the ER to Golgi transport of a subclass of N-glycosylated cargo proteins, including coagulation factors V and VIII as well as cathepsin C.[39,40] Whether VIP36 is involved as an apical lectin in cargo sorting is not yet known. It should also be pointed out that there are apical proteins that are neither present in DIGs nor glycosylated. These proteins could be linked to the rafts by protein–protein interactions or use entirely different transport routes to the apical surface. Analogously to basolateral proteins, apical sorting determinants residing in the cytoplasmic tail may also be utilized.[41]

Hierarchical order of sorting signals

Taken the complexity of the recognition principles operating in defining apical and basolateral polarity it is obvious that the sorting machinery has to make use of a hierarchy of trafficking rules. Basolateral targeting signals are usually highest in hierarchy, being dominant over raft intercalation or binding to apical lectins. For instance, a HA molecule engineered to contain a tyrosine-based sorting signal at the cytosolic tail is converted to a basolateral protein.[42] This rule explains why only a fraction of glycoproteins are sorted apically. In addition to their carbohydrates the basolateral proteins have basolateral targeting motifs. When basolateral signals are inactivated this does not always lead to apical delivery even if the protein is glycosylated. Some proteins, such as vesicular stomatitis virus (VSV) G protein or transferrin receptor are then randomly distributed apically and basolaterally.[43,44] Association with rafts requires the cooperativity of weak interactions potentially involving lipids, proteins, and sugars. Raft integrity is disturbed by cholesterol depletion resulting in breaking of clusters of GPI-anchored proteins in living cells[23] as well as reduced arrival of HA to the apical surface and concomitant missorting to the basolateral side.[45] Partitioning into rafts is necessary but not sufficient for the apical sorting of HA, suggesting that additional interactions with a raft-associated apical sorter protein are required.[32]

Small rafts cluster into apical containers

One of the most important recent insights into the mechanism of formation of apical containers is the realization that rafts are small at steady state and dispersed in the liquid disordered phase for instance in the TGN. This dispersion has been observed directly at the plasma membrane[24] (A. Pralle, K. Simons and H. Hörber, unpublished data). However, the size of rafts can be modulated by cross-linking. Using antibodies or toxins, copatching of raft lipids and proteins could be seen in morphologically discernible clusters.[30] One could envisage that *in vivo* clustering of rafts with their apical cargo in the TGN could be effectuated either lumenally or from the cytoplasmic side.

Several proteins have been identified that have raft affinity and could mediate the clustering of raft components, e.g. by functioning as raft organizers or adaptors for inclusion of cargo. Caveolin-1 (= VIP21) is an interesting candidate for clustering rafts. This protein binds avidly to cholesterol[46] and forms homo-oligomers that are incorporated into apical carriers and potentially stabilize rafts in the TGN. The formation of stabilized raft domains functioning as caveolae on the cell surface, on the other hand, seems to involve hetero-oligomerization of caveolin-1 and -2.[47] VIP17 is a tetraspan membrane protein belonging to a large family of widely expressed proteins.[48] VIP17 itself is expressed in kidney and in oligodendrocytes. In MDCK cells the protein is localizing to the TGN, to post-Golgi transport containers and to the apical plasma membrane. Recent results demonstrate that the protein is involved in apical delivery. Decreasing the expression level of VIP17 leads to pile up of apical cargo, both secretory and membrane proteins, in the Golgi complex. This proteolipid could therefore function directly in the formation of apical containers (K.-H. Cheong and K. Simons, unpublished data). Although rafts nucleate on the lumenal side of the bilayer also cytosolically oriented peripheral membrane proteins are needed in apical transport. An apically localized phospholipid-binding protein, annexin XIIIb, is found in rafts and involved in the formation of apical transport vesicles in the TGN, potentially also at later stages of transport.[49] The apical targeting of annexin XIIIb is determined by a 42-amino acid N-terminal stretch of the protein as its isoform annexin XIIIa is localized basolaterally and differs from annexin XIIIb only by the lack of this sequence (S. Lecat, F. Lafont and K. Simons, unpublished data).

Delivery of apical and basolateral cargo from the TGN

How the sorted apical and basolateral cargo is packaged into transport containers in the TGN for surface delivery is not yet known. For the basolateral proteins one could surmise that coats link the cytosolic tails together. Whether basolateral proteins with different sorting signals are segregated into one or different containers is also not clear. Somehow the regions of the TGN containing the basolateral cargo have to be released, possibly by dynamin-like molecules yet to be identified. Similar scenarios must exist for the release of the apical containers from the TGN. Obviously because most of the interactions take place within the bilayer domain or lumenally the bending of the clustered raft regions into apical containers could be mostly by intercalation of proteins such as VIP21 or VIP17 into the bilayer. Recent results following green fluorescent protein-tagged apical or basolateral cargo suggest that the containers are not only small vesicles but also larger tubulo-vesicular structures undergoing continuous fission and fusion during transport along microtubules.[50] Microtubule motors are known to connect to both the apical and the basolateral containers to ferry them from the TGN across the cell.[51,52] Whether these containers make stop-overs in endosomes on their way to the plasma membrane is not yet excluded.[53,54]

Docking and fusion of apical and basolateral containers

Ikonen *et al*[55] showed that the basolateral containers carrying VSV G protein used the NSF/SNAP/SNARE (*N*-ethylmaleimide sensitive factor/soluble NSF attachment protein/SNAP receptor) mechanism for docking and fusion. The Rab8 GTPase was connected to the delivery mechanism.[56] Recent results demonstrate that a Sec6/8 complex is recruited to the regions of cell–cell contact marked by E-cadherins and specify basolateral docking sites in MDCK cells.[57] This same Sec6/8 complex (the exocyst complex) marks the delivery of Sec4p-containing vesicles at the yeast bud in *S. cerevisiae*.[58] Sec4p is the homolog of Rab8 in yeast. Thus, the yeast post-Golgi pathway to the bud seems to be the homolog of the basolateral route in epithelial cells.

The early work on TGN to apical surface transport left the issue of the involvement of a SNARE-dependent docking and fusion mechanism open.[55,59] More recent work has demonstrated that although docking and fusion of apical containers in MDCK cells is NSF-independent the SNARE machinery components TI-VAMP, syntaxin 3, α-SNAP and SNAP23 are involved.[60,61] Recent studies in our lab have confirmed these results and also demonstrated that TI-VAMP and syntaxin 3 are raft-associated, i.e. they float in DIGs (F. Lafont and K. Simons, unpublished data). Hence, it seems likely that the apical delivery bears similarities to other membrane docking and fusion events.

Implications of the raft concept

Although the raft hypothesis was originally put forward to explain apical sorting in polarized MDCK cells it has now emerged as a key organizing principle functioning in a multitude of trafficking pathways where membrane lipid composition is compatible with raft formation, in most if not all cell types. Raft domains have been identified in the membranes of, e.g. *Drosophila*[62] and *Dictyostelium*.[63] In addition to intracellular trafficking, rafts can be applied in other dynamic assemblies of proteins requiring spatially and temporally restricted interactions, e.g. in cell signalling or adhesion. One of the first analogies was to extend the principles governing polarized exocytic transport in epithelia to the targeting of proteins to the polarized surface domains of neuronal cells.[64] There are several examples of apical proteins that are delivered to the axons in neurons whereas many basolateral markers are destined to the neuronal somatodendritic domain.[65] However, as for epithelial protein sorting apical/axonal proteins can turn into basolateral/somatodendritic proteins, emphasizing the flexible characteristics of the sorting signal hierarchies. Recent data show that some parallels can also be drawn regarding the sorting principles, e.g. rafts play an essential role in the axonal sorting of a GPI-anchored protein.[66] Nonetheless, this does not exclude that more than one route or one type of a container transports proteins to the axons.[67]

Interestingly, there are several recent suggestions that the involvement of rafts in neuronal functions may prove critical for the understanding of the pathology related to neurological diseases. For example, reports on Alzheimer's disease associated amyloid precursor protein (APP) reveal that a fraction of APP is associated with cholesterol–sphingolipid microdomains and identify rafts as the domain where intramembranous cleavage of APP to generate the amyloidogenic Aβ fragment occurs.[68–70] Also the GPI-anchored prion protein (PrP^C) and its scrapie isoform (PrP^{Sc}) have been localized to rafts and PrP^C → PrP^{Sc} transformation, the central causative event in prion disease, takes place in these domains.[71,72] A further implication of the biological relevance of cholesterol–sphingolipid rafting is provided by conditions that result from imbalances in raft lipid distribution, e.g. certain types of Niemann–Pick disease[73] where disturbed intracellular trafficking of raft lipids is accompanied by severe neurological defects.

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