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Lipid-dependent protein sorting at the trans-Golgi network ☆

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ABSTRACT

In eukaryotic cells, the trans-Golgi network serves as a sorting station for post-Golgi traffic. In addition to coat- and adaptor-mediated mechanisms, studies in mammalian epithelial cells and yeast have provided evidence for lipid-dependent protein sorting as a major delivery mechanism for cargo sorting to the cell surface. The mechanism for lipid-mediated sorting is the generation of raft platforms of sphingolipids, sterols and specific sets of cargo proteins by phase segregation in the TGN. Here, we review the evidence for such lipid-raft-based sorting at the TGN, as well as their involvement in the formation of TGN-to-PM transport carriers. This article is part of a Special Issue entitled Vesicular Transport.

1. Introduction

In cells of eukaryotic organisms, the trans-Golgi network (TGN) is described as a major sorting station for newly synthesized proteins and lipids [1,2]. This paradigm, originating in the early 1980s, was based on studies demonstrating that lysosomal enzymes depart from the late Golgi to the endosomes and on observations of the trafficking routes of different virus glycoproteins in polarized epithelial cells. Landmark experiments included infection of epithelial Madin–Darby canine kidney (MDCK) cells – a common model system to study plasma membrane (PM)-destined trafficking – with either of two viruses: influenza virus, budding from the apical membrane, and vesicular stomatitis virus (VSV) using the basolateral side of the cell [3]. When traffic in the cell was blocked by low temperature, it was found that the influenza virus protein neuraminidase (NA) was removing sialic acid (a post-translational carbohydrate modification) from the VSV G protein [4,5]. This result suggested that on their way to the different domains of the PM, NA and G protein share a common compartment, which was identified as the late-Golgi apparatus [5,6]. At the same time, it was discovered that the two viral proteins destined for different plasma membrane domains are sorted before they reach their destinations, with the Golgi apparatus as an obvious choice for the sorting compartment [7–9]. This common sorting station was termed the trans-Golgi network and it was postulated to play a central role in the sorting and polarized trafficking of biosynthetic cargo [1]. Concurrently, lipid sorting at the TGN was addressed. Using lipid analogues fed to cells and later metabolized in the Golgi apparatus, it was deduced that biosynthesized NBD-glucosylceramide was preferentially sorted at the TGN to the apical domain of the plasma membrane away from the lipids targeted to the basolateral domain [10]. These initial experiments, as well as subsequent years of research, raised the question of the mechanisms of sorting of different cargoes into their cell surface-targeted pathways.

In the classical model of recognition and sorting, specific cargo proteins are sorted by means of various coats and their associated adaptor proteins, which have a central role both in the segregation and addressing of cargo to the proper routes. In coat-mediated sorting, coat proteins (supported by specific partners, such as BAR domain-containing proteins) are responsible for membrane bending and the formation of a carrier [11–13]. Such mechanisms operate on the vacuolar, endosomal and basolateral trafficking routes from the TGN [14–19]. However, neither coat nor adaptor proteins were found to facilitate the transport of transmembrane and glycosylphosphatidylinositol (GPI)-anchored proteins from the TGN to the yeast plasma membrane or the apical cell surface of epithelial cells. These observations led to a complementary concept of sorting, cargo recruitment, and carrier formation of plasma membrane destined cargo, based on its interactions with lipids, as proposed by the lipid raft hypothesis [20,21].

In this review we will summarize the role of lipid rafts in the sorting and trafficking of proteins and lipids from the Golgi apparatus to the cell surface, focusing on mechanisms of raft clustering and transport carrier generation.

2. Lipid rafts as sorting platforms

A lipid based system for the preferential sorting of cargo without involvement of coats and adaptor proteins was proposed to be

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based on lipid rafts [20,22]. The lipid raft concept postulates that biological membranes are heterogeneous in their lateral composition, forming assemblies based on preferential associations between sphingolipids, sterols and specific proteins. According to the current view, lipid rafts are highly dynamic and their size can vary from small, short-lived, nanoscale assemblies to more stable membrane domains. Smaller rafts can temporarily coalesce into larger domains through lipid-lipid, protein–protein and protein–lipid interactions, and these stabilized domains have functional and lateral sorting potential [23–25]. Formation of such sphingolipid-rich domains was proposed to be fundamental for the differences in lipid composition of the apical and basolateral domains of the plasma membrane of epithelial cells [20]. This point of view was later expanded and lipid rafts were proposed to be directly involved in apical sorting of proteins at the TGN, based on the affinity of apically destined cargo for glycosphingolipids and cholesterol microdomains [21,26]. Further support for this postulate comes from observations that apical transport in epithelial cells is highly susceptible to cholesterol depletion or blocked sphingolipid synthesis [27–29].

3. Lipid rafts and trafficking to the apical membrane

Strong evidence supporting the hypothesis of lipid raft involvement in the TGN-to-PM trafficking came from studies in cultured epithelial cells, often MDCK. In these cells, apical delivery of influenza virus hemagglutinin (HA) was slowed upon cholesterol depletion by perturbation of synthesis (lovastatin) or extraction by chelation (methyl-β-cyclodextrin), whereas neither the transport of the basolaterally sorted vesicular stomatitis virus G protein nor ER-to-Golgi transport of either protein was affected. Additionally, HA was missorted to the basolateral domain of the plasma membrane. The decreased rate of transport was accompanied by increased susceptibility of HA to detergent extraction at 4 °C, i.e. reduced association with detergent resistant membranes (DRMs) [30]. The same missorting effect of cholesterol depletion was also observed for the apically secreted glycoprotein gp-80 [29].

More examples came from GPI-anchored proteins, which are exclusively lumenally exposed membrane proteins, and as such cannot interact directly with cytosolic components of the coat and adaptor-based sorting machinery [31]. Cholesterol depletion from culture serum was shown to decrease apical expression of GPI-anchored protein gD1-Daf (decay accelerating factor) in MDCK cells and similar results were obtained for CD14 surface expression in human monocytes [32,33]. However, in Fischer rat thyroid cells, where the majority of GPI-anchored proteins are delivered basolaterally, cholesterol depletion did not affect the apical delivery of GPI-anchored placental alkaline phosphatase (PLAP) and the chimeric neurotrophin receptor–placental alkaline phosphatase (NTR–PLAP) [27]. Additionally, some basolateral GPI-anchored proteins partition into DRMs, reflecting their association with raft lipids. Taken together, these observations suggest that such association has functional significance for plasma membrane trafficking of apical GPI-anchored proteins, but nevertheless is not sufficient as an apical sorting criterion [34–36]. At this point it has to be mentioned that sterol depletion is routinely achieved with the sterol-binding drug cyclodextrin. However, such large-scale depletion of cholesterol, not to mention any off-target lipid extraction, of live cells may cause unexpected secondary effects, therefore special caution is recommended when interpreting such experiments [37].

The situation with GPI-anchored proteins becomes even more complex in light of the observation that extensive remodeling of their anchors occurs after attachment to the protein [38]. This remodeling includes the deacylation from inositol carried by the post-GPI attachment protein 1 (PGPA1) in the ER and the replacement of unsaturated fatty-acids present in free GPIs by the saturated ones most common in peptide-linked anchors, performed by the Golgi apparatus enzymes PGPA2 and PGPA3 [39]. This modification is required for the association of GPI-anchored proteins with lipid rafts and provides an alternative explanation to the hypothesis that GPI-anchored proteins become detergent insoluble in the Golgi complex because lipid rafts are assembled there [40,41]. Varying susceptibility of GPI anchors to the remodeling enzymes might explain differences in the strength of association of different GPI-anchored proteins with lipid rafts [40]. Indeed, the trafficking of proteins was shown to be dependent on their specific GPI attachment sequences. Reporter constructs with fluorescent protein fused to the GPI attachment signals of apical or basolateral GPI-anchored proteins were targeted differently in MDCK cells and differed also in their propensities to form oligomers [42]. However, in cholesterol loaded MDCK cells, basolaterally targeted GPI-anchored proteins were found to form oligomers and were partially rerouted to the apical surface [35,42].

Recent results have strengthened the role of lipid rafts in sorting to the apical membrane in MDCK cells. Lipidomic profiling of polarizing MDCK cells has revealed a dramatic change in the sphingolipid pattern during the course of polarization. Sphingolipids in non-polarized cells are dominated by sphingomyelin, the level of which decreases during polarization as it is replaced by a complex glycolipid, the pentasaccharide Forssman antigen. This shift is accompanied by an increase in fatty acid chain length and hydroxylation of the sphingolipids. All of these changes are in agreement with the necessary rearrangements that must be introduced into the plasma membrane architecture for the formation of a lipid raft-rich apical membrane to produce a polarized epithelial cell [43]. The specific role of the Forssman glycolipid has been illuminated by the recent finding that in MDCK cells, knockdown of galectin-9 binding to this glycolipid leads to a serious breakdown of surface polarity [44]. There are likely to exist multiple glycolipid–lectin couples specific for different cell types, since the Forssman glycolipid is not ubiquitously found in every cell type [45].

One issue with the glycolipid-assisted lipid raft concept in the generation and maintenance of the apical membrane has been the lack of genetic evidence for this model. However, recently it was established that glycosphingolipids indeed play such a role in *Caenorhabditis elegans*. A combination of genetic screens, lipid analysis and imaging methods was used to show that these lipid raft components mediate apical sorting in the gut of this animal [46].

4. Lipids in trafficking to the plasma membrane in yeast

Due to the variety of available tools and relative simplicity of genetic manipulations, the yeast *Saccharomyces cerevisiae* has proven to be an excellent system to study intracellular protein and lipid trafficking, and therefore has been fundamental in deciphering trafficking-related processes [47].

In yeast, most plasma membrane proteins, including all GPI-anchored proteins, were found to be associated with lipid rafts (as reflected by their inclusion in detergent-resistant membranes). Among many, the list includes: the H^+-ATPase Pma1p, the most abundant plasma membrane protein in yeast, tryptophan permease Tat2p, uracil permease Fur4p, permeases Hxt1p, Hxt2p and Gap1p, GPI-anchored cell wall protein Gas1p and the lipid raft model chimeric construct protein, FusMidp [48–57]. Some membrane proteins, e.g. Gap1p, associate with DRMs at the level of the Golgi apparatus, whereas GPI-anchored proteins like Gas1p seem to associate with sterol and sphingolipids earlier, already in the ER. However, vacuolar and ER resident proteins are excluded from DRMs [48,55].

Significantly, proper localization and trafficking of proteins to the plasma membrane was shown to be dependent on raft lipids. A well-studied example is Pma1p. This protein requires ongoing sphingolipid synthesis for its proper plasma membrane targeting and stability at the cell surface [48,49,58]. With sphingolipid synthesis blocked (by a temperature-sensitive mutant of a subunit of the serine

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palmitoyltransferase complex *lcb1–100*, or upon inhibition of sphingoid base synthesis with myriocin, or inhibition of ceramide synthesis with fumonisin B), Pma1p cannot properly associate with lipid rafts and is accumulated intracellularly or missorted to the vacuole for degradation [49,53]. In addition, an allelic version of Pma1p, *pma1–7*, which loses lipid raft association at the restrictive temperature, was shown to be missorted to the vacuole. Interestingly, this missorting could be rescued by the overexpression of Ast1p, which restores the plasma membrane localization of Pma1p [59]. It was shown that Ast1p directly interacts with Pma1p promoting its oligomerization and proper insertion into lipid rafts at the level of Golgi apparatus. However, later Pma1p was found to oligomerize in a ceramide synthesis-dependent manner already in the ER [49,58]. The detailed studies of Pma1p trafficking with respect to lipid metabolism revealed that synthesis of lipids containing very long fatty acid chains (26 carbon atoms long, C26) rather than ceramides or sphingolipids *per se* is essential for lipid raft association and proper delivery of Pma1p to the plasma membrane [53,54].

A visual screen involving FusMidp, a chimeric protein used as a model lipid raft-associated plasma membrane cargo, revealed that both ergosterol synthesis and sphingolipid synthesis are essential for its proper plasma membrane targeting. Without the *ERG4* or *ERG6* genes, encoding enzymes involved in ergosterol synthesis, or *ELO3* and *SUR2*, both involved in sphingolipid synthesis, FusMidp was observed to accumulate in the Golgi apparatus or become missorted to the vacuole [60]. *ELO3* involvement in FusMidp trafficking resembles closely the Pma1p situation, since this gene is involved in the conversion of C24 to C26 fatty acids incorporated into sphingolipids [61,62]. Sphingolipid synthesis was also shown to be essential for transport of Fur4p and Gap1p to the plasma membrane [56,63].

GPI-anchored proteins in ergosterol mutants in yeast are still efficiently transported from the ER to the Golgi apparatus, however they lose their DRM association and some of them become missorted to the vacuole [64,65]. Ergosterol is also required for transport of Tat2p to the plasma membrane, being missorted to the vacuole in *ERG6* or *ERG2* mutants [51,66]. An ergosterol requirement for cell surface transport has also been documented for Fus1p, an integral membrane protein required for mating between yeast cells [67]. In the case of Fur4p, defective synthesis of both ergosterol and unsaturated fatty acids (UFAs) caused its mislocalization to the vacuole [57].

Apart from the raft forming lipids, other lipid requirements for plasma membrane delivery of membrane proteins in yeast have been reported. Several proteins rely additionally on phosphatidylethanolamine (PE) for trafficking through the secretory pathway, including Gap1p, Fur4p, the proline transporter Put4p, the maltose transporter Mal6p and the arginine permease Can1p [68,69]. However, Pma1p and Hxt1p do not require PE for plasma membrane delivery, suggesting a specific requirement for PE in the case of certain proteins only. How the depletion of PE mechanistically results in the missorting of plasma membrane-destined proteins is not known. Based on altered sensitivity of Can1p to extraction with detergents in PE-depleted cells, PE could be involved in the assembly of a specific set of membrane microdomains in yeast [70]. The fact that Pma1p specifically requires C26 long fatty acid-containing lipids and the dependence of Fur4p on both ergosterol and UFAs suggests additional fine tuning of the lipid environment of the TGN to be essential for proper sorting.

GPI-anchored proteins seem to have a peculiar role in this scheme. As mentioned, in yeast they become associated with lipid rafts starting in the ER, and this seems to be crucial for their proper plasma membrane delivery. However, GPI-proteins themselves are also required for the proper transport of some transmembrane proteins to their destination. It was shown that when GPI modification of proteins was blocked using the temperature dependent mutant *gwt1–10*, two proteins Tat2p and Fur4p, which are not GPI-anchored, were retained in the ER, concomitant with a loss of DRM association [71]. Moreover, the deletion of the *GUP1* gene, encoding an *O*-acyltransferase involved in remodelling of the GPI anchor, interferes with lipid metabolism, leading to decreased stability/integrity of ergosterol-sphingolipid

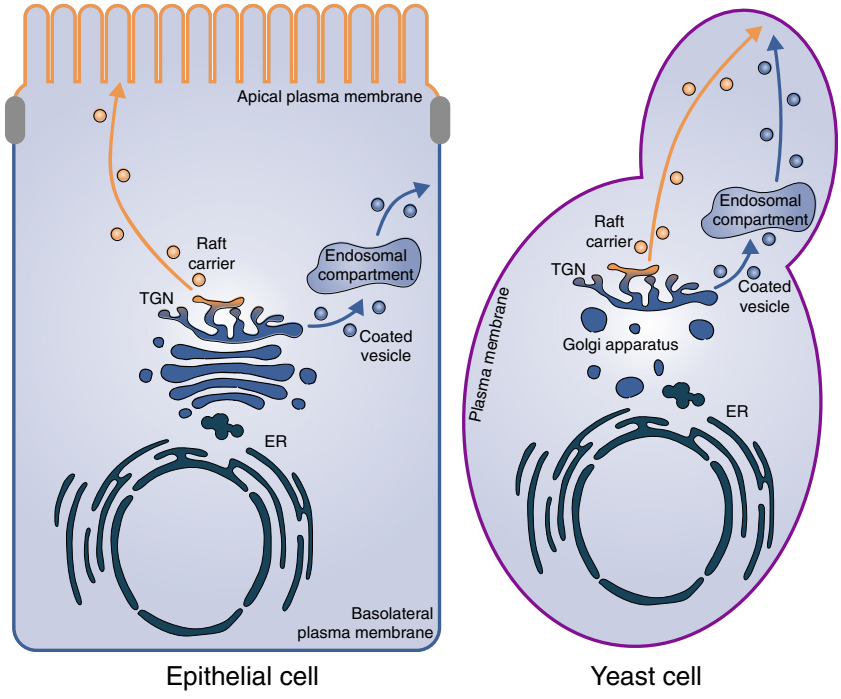

Fig. 1. Yeast share a similar organization of the late secretory pathway with highly polarized epithelial cells. The yeast plasma membrane is not organized into distinct apical and basolateral domains. Rather, two major plasma membrane bound pathways originate from the yeast trans-Golgi network (TGN), and these could be considered as analogous to apical and basolateral routes in epithelial cells. Both pathways are directed toward the growing tip of a budding daughter cell, where the exocyst machinery is localized, and both rely on the same set of SNAREs. However, one pathway is raft lipid dependent and direct, whereas the other utilizes coated vesicles and most likely an intermediate station (i.e. the endosomal system). These pathways also differ in cargo proteins transported; the raft one carries PM and GPI-anchored proteins, including cell wall proteins, while the other mainly transports periplasmic enzymes. Other pathways, including retrograde ones, are omitted in this figure. ER, endoplasmic reticulum.

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domains and also lowering Pma1p and Gas1p in DRMs [72,73]. These results suggest that in yeast, GPI-anchored proteins might have a role beyond their own specific biological functions, perhaps assisting lipid raft-based trafficking to the cell surface.

5. Raft lipid segregation at the TGN

A major prediction of lipid raft involvement in sorting and trafficking of cargo from the TGN to the PM is the sorting of lipids and proteins bound for the apical plasma membrane of epithelial cells into common carrier vesicles [20]. So far, repeated attempts to isolate the apical transport carriers in a pure form have failed. However, due to superior genetic tools and increased material yields, isolation of the yeast post-Golgi transport vesicles has been successful [74].

Analogous to apical and basolateral routes in epithelial cells, two major plasma membrane-bound pathways originate from the yeast TGN (Fig. 1). The two pathways transport different cargo proteins; one being responsible for transporting integral plasma membrane proteins e.g. Pma1p, Hxt2p and Gas1p, as well as a variety of cell wall proteins like Bgl2p and Chs3p, whereas the other transports the periplasmic enzymes, like invertase and acidic phosphatase [67,75–78]. As described earlier, cargo proteins transported via the first route rely on raft lipids for their sorting and trafficking, whereas the second route was found to be clathrin (Chc1p)- and dynamin (Vps1p)-dependent [79]. Using a tagged version of the raft protein FusMidp as bait, it was possible to immunoisolate secretory vesicles of the first pathway. Subsequent quantitative analysis of these vesicles by mass spectrometry revealed that they are highly enriched in ergosterol and sphingolipids, relatively to the donor TGN [74]. This was the first direct evidence supporting the hypothesis that plasma membrane proteins are delivered to their destination in lipid raft enriched carriers, which implies raft lipid sorting at the TGN. Moreover, spectroscopic measurements using the membrane dye C-laurdan showed that these vesicle membranes are more condensed in comparison to the donor compartment, suggesting a change in membrane packing during sorting. Further experiments with additional plasma membrane proteins as baits showed that lipid raft sorting is a generic feature of vesicles carrying transmembrane and GPI-protein cargo to the plasma membrane [80]. The sorting of sphingolipids and sterols into the post-Golgi anterograde carriers is accompanied by exclusion of these lipids from retrograde COPI-coated vesicles [81]. These processes help to establish the lipid gradient characteristic of the secretory pathway, facilitating correct trafficking toward the cell surface [82].

6. Clustering of lipid rafts

The model of lipid raft-based sorting and carrier formation assumes that the initial state, when lipid rafts exist as nanoscale entities within the surrounding lipid environment, needs to be modified by clustering of smaller assemblies into larger, more stable, functional domains, eventually leading to segregation of cargo and the formation of vesicular raft carriers (Fig. 2) [22]. This process needs to be induced because *in vivo* lipid raft clustering does not occur spontaneously, thus suggesting the requirement for additional clustering/coalescence factors [23]. Theoretically, such lipid raft clustering could be induced by a number of different means.

In the simplest possible scenario, lipid raft coalescence might be induced by driving the system above the critical concentration of raft components passively by sorting out non-raft constituents. In yeast, it was shown that phase separation could be induced for lipid mixtures extracted from whole cells, stressing their natural, non-induced connectivity that would have to be enhanced by increased concentration of raft lipids in the TGN [83]. This mechanism of lipid rafts coalescence fits well with the Golgi apparatus cisternae maturation model in yeast, where new Golgi cisternae are postulated to be

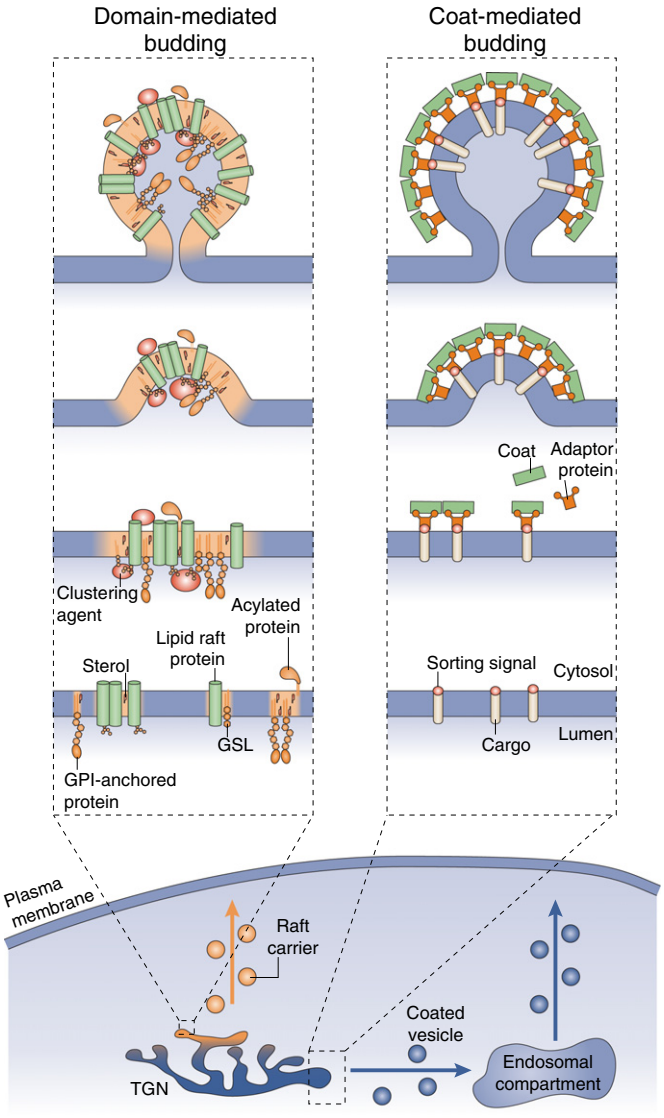

Fig. 2. Transport carrier generation based on clustering of lipid rafts and domain induced budding. Right: Cargo destined for the endosomal compartments in yeast, or the basolateral plasma membrane in epithelial cells, is sorted by adaptor and coat proteins recognizing cytoplasmic sorting signals. The generation of transport vesicles by membrane bending and subsequent budding is driven by the formation of the coat, aided by specific bending proteins (not shown). Left: Lipid rafts carrying associated cargo proteins are sorted into a lipid raft transport carrier, following their clustering into a raft platform. Raft coalescence is induced by clustering agents, which might be lumenal lectins or cytosolic peripheral proteins. The growing platform selects raft constituents and excludes non-raft cargo, sorted away by signals that are dominant over lipid raft sorting principles. Membrane bending and vesicle budding are driven by the increasing line tension between the domains, but these processes have to be additionally facilitated by auxiliary factors, like membrane bending proteins. Also the actin cytoskeleton plays the role, so does the fine tuning of lipid environment. Cytoskeletal elements important for both processes are not shown. GSL, glycosphingolipids. Figure adopted and modified with permission from [24].

generated *de novo* from the ER and then converted into cis- and subsequently medial- and trans-cisternae [84–88]. In this model, a constant flow of cargo is continuously synthesized and provided from the ER by vesicles supplying membranous compartments, which later gradually change their identity. Non-raft cargo destined for intracellular compartments (endosomes and vacuole) would be sorted away by signals that are dominant over a lipid raft sorting mechanism (e.g. ubiquitylation or adaptor proteins and clathrin). This scenario is further supported by the fact that cargo from the clathrin and dynamin-dependent route in yeast is re-routed to the lipid raft-dependent route upon deletion of genes that are required for the

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endosomal route [79]. The same effect as for clathrin and dynamin was observed when genes encoding proteins from the late endosomal pathway, like *PEP12* – the t-SNARE of late endosomes or *VPS4* – AAA-ATPase involved in multivesicular body sorting, were deleted [78,79].

Cisternal maturation has also been postulated to play a major role in transport through the Golgi apparatus in mammalian cells [89]. However, several alternative models have been proposed to explain Golgi apparatus structure and function. These include models based on vesicular transport, cisternal progenitors and "rapid partitioning", where cargo introduced into the Golgi is postulated to mix rapidly and partition into lipid-based export domains [90–92]. Further, observations of tubular connections between the cisternae suggest direct cisternal contact [93]. Thus, there is no consensus on how the Golgi apparatus functions [94]. In this context, the question arises as to the mechanism and regulation of active clustering of small lipid raft assemblies into export domains in the TGN that would eventually give rise to secretory vesicles.

An alternative mechanism of lipid raft coalescence was proposed to be based on "lectin-like" clustering agent. It exploits the crucial role of specific protein glycosylation in plasma membrane-bound trafficking in yeast and apical trafficking in epithelial cells, and the fact that most sphingolipids are glycosylated [22,52,95,96]. In polarized epithelial cells, lectins are known to be involved in the function of the early secretory pathway, where they were found to have a role in proper trafficking of *N*-glycosylated cargo [97–100]. The recent identification of galectin-9 as an important player in apical membrane biogenesis strengthens the evidence for a raft-based sorting mechanism in MDCK cells. Galectin-9 binds to the Forssman glycolipid, which might be instrumental in driving raft-based sorting in the TGN [44]. However galectins are secreted from cells by a non-classical mechanism that bypasses the Golgi complex, raising the question of how they can enter the Golgi apparatus to perform the lipid raft clustering function. One possibility is that they re-enter the secretory pathway upon endocytosis [44,101]. In this scenario, galectin-9 would partially dissociate from the Forssman glycolipid in the low pH environment of the endosomes that it traverses before reaching the Golgi, and thus be able to nucleate raft coalescence again. Similar to the galectin-9-Forssman antigen paradigm, galectin-4 binds to a glycolipid sulfatide in enterocyte-like HT-29 cells, and is involved in apical sorting [102]. Thus, there may be similar lectin-glycolipid couples that function in apical biogenesis in other cell types, making the entire galectin family a strong candidate for raft lipid clustering agents [44,102]. Contrary to the epithelial cells, yeast does not have any galectins or galectin-like proteins encoded in their genome [103]; however, a number of other yeast lectins are known, including flocculins and agglutinins. Interestingly, many of them are GPI-anchored proteins [104].

Lipid rafts are primarily organized from the exoplasmic membrane leaflet but they are matched by domains on the cytoplasmic side [105–109]. Thus, clustering of rafts by cytoplasmic agents is also possible, with annexins as candidates. Annexin 2 and annexin 13b facilitate apical transport in MDCK cells and both preferentially associate with cholesterol-rich domains [110–112]. Moreover, annexins were shown to form large, two-dimensional ordered arrays, with annexin 2 also having lipid segregation potential [113,114].

Myelin and lymphocyte (MAL) proteolipids, membrane proteins associated with lipid rafts and having a role in apical transport, might also serve a role as clustering agents. VIP17/MAL, which cycles between the Golgi apparatus and the apical cell surface, was shown to form oligomers, which may induce raft clustering [115–117]. Moreover, spontaneously formed or antibody-cross-linked MAL clusters show lateral concentration of sphingolipid raft markers and exclusion of a fluorescent analogue of PE, making MAL a key component in the organization of membrane domains and sorting platforms [118].

Additionally, cargo protein oligomerization might amplify their affinity for lipid rafts and enhance clustering of lipid raft platforms. As mentioned, ceramide-dependent Pma1p oligomerization, further facilitated by Ast1p, was crucial for the association with DRMs and proper PM delivery. In the case of GPI-anchored proteins in polarized epithelial cells, the GPI-anchor itself is insufficient for proper delivery and only upon oligomerization of GPI-proteins do they reach their apical destination [36]. Moreover, it was shown that this oligomerization principle is not used by apically destined non-raft proteins [119]. Neither *O*- nor *N*-glycan motifs of apical GPI-proteins were directly involved in their oligomerization; however their apical delivery was perturbed by tunicamycin, an inhibitor of protein *N*-glycosylation, combined with cholesterol depletion. It was postulated that some putative, proteinaceous lipid raft-associated *N*-glycosylated interactor may be involved in the recognition and oligomerization of apical GPI-anchored proteins [120].

7. Lipid rafts and transport vesicle generation

Lipid raft clustering also provides a mechanism for the generation of specific transport carriers at the TGN (Fig. 2). The mismatch in biophysical properties (e.g. more ordered versus less ordered) between the growing lipid raft "platform" and surrounding non-raft environment is energetically unfavorable, and results in a line tension along the domain boundaries. This tension increases with the length of the domain boundary, which grows with the size of the domain. The tension can be decreased by reducing the length of interphase boundaries. In three-dimensional systems this may be achieved by budding out the lipid raft portion of a membrane, and this occurs once the line energy becomes greater than the energy costs of membrane bending in the forming bud [121,122]. This process of small, ordered phase domains budding out and eventually pinching off from the donor membrane was indeed shown in artificial systems [123,124]. The growing curvature of a membrane close to the demixing point (phase separation) further induces lipid sorting based solely on underlying connectivity of sorted lipids and is greatly amplified by lipid clustering [125]. Since the curvature of a membrane can also drive protein sorting, a growing bud can generate a feedback system whereby curvature-preferring proteins would be recruited to a growing lipid raft platform, further increasing the propensity to generate curvature [126–129]. Once a curved membrane is generated, phase separation into membrane tubes can trigger membrane fission arising from the difference in elastic constants between the domains [130,131].

If the processes of bilayer bending, budding, and vesicle formation were only membrane-driven (i.e. propelled exclusively by line tension between the membrane phases), raft domains in the range of several hundreds of nanometers would be required [122]. Therefore, *in vivo* these processes are probably facilitated by auxiliary factors, likely proteins [125,132]. BAR domain-containing proteins could be involved in the generation of membrane curvature, as Rvs161p, the amphiphysin-like lipid raft protein, is required for the plasma membrane delivery in yeast [60,133]. Protein induced curvature has also been shown to be generated by clustering of glycosphingolipids by their toxin ligands, where it takes the form of tubular invaginations from the plasma membrane [134,135]. In this scenario, the final force for vesicle scission and endocytosis involves the actomyosin cytoskeleton in the absence of coat proteins or dynamins [136]. The actin cytoskeleton has been postulated to be involved in membrane-lipid raft dynamics, acting as a scaffold or nucleator for driving lipid raft coalescence [137,138]. Indeed, actin polymerization was demonstrated to be critical for the lateral organization of apical and basolateral proteins in the Golgi apparatus [35].

For clarity, raft carrier formation is described here as a separate, subsequent event to lipid raft clustering. However, it must be stressed, that in the dynamic environment of the TGN, these processes are very likely interconnected both spatially and temporally, both affecting the progress and influencing the outcome. This interdependence might

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become a major obstacle in further investigation of TGN events, specifically in attempts to reconstitute the process in simplified, *in vitro* systems. It is probable that different types of factors (i.e., clustering, bending and pulling agents) are involved in the result of lipid-driven domain clustering and budding, with specific machineries employed by different cargoes.

8. Regulation and fine tuning of lipid based vesicle generation

Assuming that effective vesicle budding and scission requires a system close to the demixing and phase separation point, the lipid composition at the site of carrier generation would need to be fine-tuned to regulate and assist these processes.

For example, phosphatidic acid (PA) resulting from the action of Golgi-associated phospholipase D has a role in membrane bending and secretory vesicles budding mediated by BAR-domain proteins, suggesting its mechanical involvement in carrier generation [139,140]. This is further supported by the fact that this lipid was found at elevated levels in secretory vesicles in yeast [74]. Also mentioned earlier, asymmetric lipids containing very long fatty acid chains (C26) necessary for the proper trafficking of Pma1p were shown to have a role in stabilizing highly curved membrane structures potentially by lowering the energy required for bending by coupling bilayer leaflets through fatty acid chains interdigitation [141,142]. Similarly, in yeast lipid raft-dependent secretory vesicles, sphingolipid species that had specific asymmetry provided by C26 and C24 fatty acids chains were found to be enriched. These could have a direct role in carrier generation [80]. For the maintenance of optimal membrane curvature essential for Fur4p delivery, ergosterol and unsaturated fatty acid-containing lipids were proposed to play a role [57].

It is also known that a certain lipid balance is essential for the proper function of secretion in yeast. Disruption of the function of the *SEC14* gene, which encodes the phosphatidylinositol/phosphatidylcholine transfer protein, causes a block in secretion and abnormalities in intracellular transport [143,144]. On the other hand, Kes1p, a member of the oxysterol binding protein family has a role in negative regulation of Sec14p-dependent secretion [145]. Also the recruitment and activation of phosphatidylinositol 4-kinase IIIβ (PI4KIIIβ), resulting in the production of phosphatidylinositol 4-phosphate (PI4P), which is a marker for TGN, plays a crucial role in regulation of carrier budding mediated by tuning the membrane lipid environment. Studies demonstrate that PI4P and its effectors connect vesicle budding with lipid dynamics, ensuring that the appropriate membrane composition is established before a transport vesicle buds from the Golgi. PI4P promotes the recruitment of a variety of lipid-transfer proteins, including OSBP1, CERT and FAPP2. CERT delivers ceramide from its site of synthesis in the ER to the Golgi apparatus, supporting synthesis of sphingolipids, and OSBP1 is linked to sterol metabolism [146–148]. Moreover FAPP2, which was shown to be involved in the transport of apical cargo in polarized MDCK cells, has membrane tubulating potential achieved by a wedge-shaped motif penetrating a bilayer [149–151]. Involvement of Sec14 and FAPP2 proteins in vesicular transport is described in more detail elsewhere in this issue [152,153].

Altogether these facts suggest that in addition to lipid rafts and their associated components, other factors, including fine-tuning lipid levels are involved in cargo sorting and carrier formation at the TGN.

9. Outlook

The role of lipid rafts in the sorting and generation of post-Golgi transport carriers is increasingly supported by studies on epithelial MDCK cells and yeast. The route to the cell surface in yeast seems to be a direct one, while in MDCK cells, endosomes have also been implicated to be stations along the pathway [40]. Clearly, endosomes are involved in recycling apical and basolateral proteins and lipids. Delineating the pathways and quantifying the routes that different cargoes take to the cell surface remains a difficult issue in membrane trafficking research. One reason is that blocking one pathway by inhibitors or mutants of machinery proteins often leads to rerouting of cargo to an alternative route. Until a methodology is developed that can overcome these obstacles, these issues will not be resolved. For lipid raft mediated sorting and transport carrier formation, the challenge now will be to analyze the process mechanistically by reconstitution *in vitro*. Taking this approach we can begin to dissect the mechanisms involved in raft based sorting by biochemical and biophysical means.

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