Lipids and the exocytotic machinery of eukaryotic cells

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The molecular machines that drive protein transport through the secretory pathway function exert their activities on the surfaces of membrane bilayers. It is now clear that the various lipid components of these bilayers play direct and versatile roles in modulating the activity of proteins that either themselves constitute core components of the membrane trafficking machinery, or represent proteins that regulate such core components.

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Abbreviations

ARF	ADP-ribosylation factor
CAPS	calcium-activated protein for secretion
DAG	diacylglycerol
DCV	dense core vesicle
ENTH	epsin amino-terminal homology
GAP	GTPase-activating proteins
IEV	intracellular enveloped virion
OSBP	oxysterol-binding protein
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PH	pleckstrin homology
PI	phosphatidylinositol
PIP_2	PI 4,5-bisphosphate
PITP	phosphatidylinositol transfer protein
PKD	protein kinase D
PLD	phospholipase D
PtdOH	phosphatidic acid
PS	phosphatidylserine
SCAP	SREBP-cleavage activating protein
SLMV	synaptic-like microvesicle
SREBP	sterol regulatory element binding protein
TGN	*trans*-Golgi Network

Introduction

The past 30 years have witnessed remarkable advances that arrive at a detailed molecular understanding of how cells regulate and execute the myriad of membrane trafficking reactions. The sum of these reactions defines a major pathway for how cells generate and maintain their highly complex internal organization. Throughout this period, the discovery and characterization of proteins required for specific membrane trafficking steps along the secretory pathway was emphasized [1,2]. Our understanding of the roles played by lipids in this process has evolved more slowly.

The first clear demonstration that lipids are not passive constituents of the membrane trafficking mechanism, but are active and intimate partners that regulate these molecular events, came from studies in yeast. It was demonstrated that a phosphatidylinositol (PI) transfer protein (PITP; Sec14p) is essential for protein trafficking from the yeast *trans*-Golgi network (TGN) [3], and that this essential requirement could be overcome by inactivation of a specific pathway for phosphatidylcholine (PC) biosynthesis [4]. The explicit discussion that proper coordination of the metabolism of both inositol- and choline-containing lipids is essential for TGN-derived vesicle biogenesis was thereby initiated [4,5]. Since then, the accumulated evidence clearly makes the case that several lipids enter into intimate collaborations with proteins to drive specific stages in membrane transport. In this regard, the involvement of phosphoinositides in membrane trafficking has been discussed at length in several recent reviews [6,7]. To broaden discussion of this topic, here we review recent evidence emphasizing the roles of other lipid species in regulating secretory pathway function.

Lipids and sorting of cargo at the endoplasmic reticulum

The first step in the membrane trafficking itinerary of secretory cargo is packaging into ER-derived vesicular carriers that ferry material from the ER to the Golgi complex. Although it is appreciated that there is an active quality control mechanism that ensures only properly folded protein cargo is incorporated into these vesicles [8], the popular view has long held that properly folded proteins are packaged into common vesicular carriers at the level of the ER, irrespective of the ultimate destination of the cargo molecules. Recent studies indicate that this view is too simple, and that specific lipids play novel and determining roles in cargo sorting/packaging reactions at the level of the ER.

Sterol regulatory element binding proteins (SREBPs) exit the ER as a complex with SREBP-cleavage activating protein (SCAP), and this complex is a substrate for Golgi-localized proteases that cleave SREBP in two steps, to generate a transcription factor that activates expression of lipid biosynthetic genes [9,10]. Recent work demonstrates trafficking of SCAP from the ER is regulated by sterols *in vivo* and *in vitro* [11]. Specifically, sterol depletion

stimulates the packaging of SCAP into ER-derived vesicles and impressive stimulatory effects are also recorded when SREBP packaging into vesicles is scored in the presence of SCAP under conditions of sterol depletion. Because this sterol effect is specific (i.e. trafficking of other secretory cargo proteins is unaffected), a general effect on ER-derived vesicle biogenesis is unlikely.

Although it remains possible that SCAP–SREBP is mobilized from the ER by a dedicated class of vesicle (see below), it seems more plausible that sterols either bind SCAP and prevent its association with the vesicle budding machinery or that sterols stabilize the association of SCAP with an ER component that prohibits incorporation of SCAP into transport vesicles [11]. One attractive mechanism for such a retention is sterol binding effecting a conformational change in SCAP so that it is registered as an inappropriate cargo for transport from the ER by the resident quality control machinery. In support of this possibility, SCAP undergoes a conformational change when cholesterol is introduced into ER membranes [12].

Another example of metabolic control of protein exit from the ER comes from the study of the egress of lipid-modified proteins from the ER. Glycosylphosphatidylinositol (GPI)-anchored proteins exit the yeast ER in vesicular carriers that are biochemically distinct from those employed by other secretory cargo [13••]. A specific requirement for this particular ER exit pathway is the active biosynthesis of sphingoid bases that serve as metabolic precursors to ceramide [14]. Thus, ceramide (or one of its precursors) is somehow required for the packaging of GPI-linked cargo into a dedicated class of transport vesicles. It remains unresolved whether this sphingoid base requirement reflects a signaling pathway required for assembly of vesicle coat components in a coupled cargo selection/vesicle budding reaction, or whether sphingoid bases help form a lipid microdomain that functions as a physical platform for the coalescence of cargo with vesicle coat components.

Lipids and biogenesis of transport vesicles from Golgi membranes

The essential involvement of the major yeast PITP (Sec14p) in coordinating the interface between lipid metabolism and biogenesis of TGN-derived vesicles is clear [2–4]. A combination of genetic and biochemical data now suggest that Sec14p controls lipid metabolism to activate an essential pair of ADP-ribosylation factor (ARF) GTPase-activating proteins (GAPs; Gcs1p and Age2p) essential for budding of vesicles from yeast TGN [15•,16]. The GAP activities of Gcs1p and Age2p are stimulated by diacylglycerol (DAG) and phosphatidic acid (PtdOH) and inhibited by PC *in vitro*, a pharmacology consistent with that predicted by genetic studies [17–19]. Interestingly, these ARF-GAPs are not activated by phosphoinositides, and the pleckstrin homology (PH) domains of Gcs1p and Age2p are dispensable for *in vivo* function [15•]. The lipid activation of Gcs1p and Age2p could be imposed by an allosteric mechanism, or it might reflect lipid-driven recruitment of these ARF GAPs to their site of action on TGN membranes.

The proposed role for ARF GAPs in the vesicle budding process departs from the long-held view that ARF GAPs play a late role in the vesicle cycle (i.e. at the level of vesicle uncoating before fusion) [1]. However, newly designed *in vitro* vesicle budding assays that employ GTP in the budding reaction, rather than nonhydrolyzable GTPγS, as had been classically employed, indicate that the rate of vesicle formation is highly stimulated by ARF GAPs and that ARF GAP activity is critical for loading of cargo into nascent transport vesicles [20,21,22••]. One interpretation of these data are that ARF–GTP recruits ARF GAPs to membranes so that vesicle budding can occur, a scenario that represents a significant departure from popular models. In the case of the reconstituted ARF-GAP1-dependent mammalian vesicle budding system, it is also worth noting that ARF-GAP1 activity is stimulated by DAG and inhibited by PC [23]. Thus, ARF GAPs represent a new set of candidate effectors for lipid regulation of vesicle budding in mammals and yeast. In this regard, discovery of the Golgi-associated ARAP (ARF and Rho GTPase-activating protein) family of GAPs that exhibit both ARF GAP and Rho GAP activity, and whose ARF GAP activity is potently stimulated by PI 3,4,5-trisphosphate, foreshadow key roles for phospholipids in coordinating ARF and Rho GTPase signaling pathways [24,25].

PI 4-phosphate has been implicated as a key stimulatory lipid for Golgi function on the basis that inactivation of the yeast Pik1p PI 4-kinase levies a measurable, but modest, defect in protein transport from the Golgi complex [26,27]. What proteins are effectors of PI 4-phosphate in Golgi membranes? Two lines of inquiry indicate that members of the ubiquitous and enigmatic oxysterol-binding protein (OSBP) family bind PI 4-phosphate on Golgi membranes, and that this binding is a necessary (but insufficient) component of the mechanism by which these OSBP homologs are targeted to the Golgi [28•,29]. What role is discharged by these OSBP homologs in the Golgi membrane trafficking program? One of these PI 4-phosphate-binding proteins, Kes1p, exerts an inhibitory effect on vesicle budding from the yeast Golgi complex, as illustrated by the demonstration that loss of Kes1p function restores Golgi secretory activity and viability to Sec14p-deficient yeast mutants [30]. While the precise function of Kes1p remains to be determined, present data suggest that Kes1p interfaces with the ARF cycle, perhaps by regulating ARF GAP function [28•]. The demonstration that PI 4-phosphate is a key molecular cofactor in the activity of a negative regulator of Sec14p-dependent Golgi secretory function indicates the biology of PI 4-phosphate function on Golgi membranes is more complex than anticipated.

The involvement of protein kinase D (PKD) in stimulating vesicle budding from mammalian TGN provides a strong example where DAG provides a platform for recruitment of a component of the vesicle budding machinery, thereby creating a spatial protein landmark for a cascading recruitment of additional transport factors. PKD is a multidomain protein consisting of two cysteine-rich domains (C1a and C1b), a PH domain and a catalytic domain. The level of TGN vesicle budding activity is directly proportional to PKD activity, indicating a central involvement of PKD in this process [31,32]. PKD is a DAG-binding protein, and DAG represents a major cue for PKD recruitment to Golgi membranes [33••]. Once positioned on TGN membranes, PKD probably catalyzes recruitment of other signaling molecules via protein–protein interactions. These include PI 4-kinase, PI 4-phosphate 5-kinase, protein kinase C (PKC) η and ε isoforms, and heterotrimeric G-protein βγ subunits. The presence of other lipid-modifying enzymes on the roster of PKD interacting proteins suggests that a cascade of lipid modifications comes into play as one progresses through the vesicle biogenesis program.

A new twist on the relationship between lipids and the exocytotic machinery is apparent from demonstrations that putative aminophospholipid translocases, or 'flippases', play a role in vesicle budding from yeast membranes. Yeast express five members in this protein family, and one of these (Drs2p) is implicated in clathrin function at the level of yeast TGN [34]. Multiple deletions of genes within this family result in defects in late secretory pathway and endosomal function, consistent with a membrane trafficking function for these proteins [35,36]. The finding that phosphatidylethanolamine (PE) leaflet asymmetry in plasma membrane is compromised in yeast mutants with multiple deficiencies in this protein family, when coupled with the ability of these proteins to catalyze flipping of short-chain NBD–phospholipid analogs *in vitro*, suggests that these proteins are authentic flippases [36]. These data suggest that the aminophospholipid (i.e. PE and PS) content of the cytoplasmic leaflet of Golgi, endosomal and plasma membranes contributes to the vesicle budding competence of these membranes.

Phospholipase D

PLD catalyzes hydrolysis of the phosphodiester bond of PC to generate PtdOH and free choline. PtdOH itself exhibits signaling properties, and it is also a substrate for dephosphorylation to another signal-active lipid DAG. In mammals, there are two PLD isoforms, PLD1 and PLD2, both of which are subject to complex regulation by lipid cofactors, protein kinases and GTP-binding proteins of the ARF and Rho families [37]. Yeast exhibit one PLD and this enzyme plays a pivotal role in distinguishing between constitutive and developmentally-regulated (i.e. sporulation-specific) membrane trafficking events in budding yeast, although it is nonessential for membrane trafficking in vegetative yeast cells except under unusual circumstances [38–41]. Both mammalian and yeast PLDs are potently and obligatorily stimulated by PI 4,5-bisphosphate (PIP_2) through interaction with a conserved polybasic motif positioned close to the catalytic core of these enzymes [42•]. This activation in vegetative yeast cells is coupled to the action of four nonessential PITPs that share structural homology with Sec14p [43].

While there is considerable circumstantial evidence to suggest an essential role for PLD in mammalian vesicle trafficking, convincing proof that this is indeed the case remains elusive. Recent localization experiments indicate the existence of pools of both PLD1 and PLD2 on Golgi membranes, with a broad distribution of PLD1 throughout the organelle and a concentration of PLD2 at the rims of Golgi cisternae [44,45]. One interpretation of these data is that PLD1 plays an essential housekeeping role in maintaining structural integrity of the Golgi complex, whereas PLD2 plays a direct role in vesicle biogenesis.

Support for a role of PLD in vesicle budding from the Golgi is provided by recent studies of pox virus replication and packaging [46–49]. These viruses replicate in the cytoplasm of infected cells to form mature intracellular enveloped virions (IEVs) wrapped in Golgi-derived membranes. IEVs then travel along microtubules and fuse with the plasma membrane. Two virally encoded proteins required for the membrane-wrapping step are associated with IEV membranes and one of these (F13L) exhibits a single variant PLD catalytic motif. While the catalytic activity of this putative PLD has not yet been demonstrated and its substrate selectivity remains uncharacterized, mutation of residues within this motif that produce what are expected to be inactive alleles yield immature and non-infectious virions with wrapping defects [47]. In addition, F13L with an intact catalytic motif stimulates formation of post-Golgi vesicles in a manner sensitive to challenge with primary alcohols [48]. These collective data suggest F13L is not a PLD activator but a viral phospholipase that subverts the Golgi-derived vesicle budding process to allow the production of IEVs.

Lipids and vesicle fusion

Studies elucidating the mechanism of how neurotransmitter-loaded vesicles dock and fuse to the presynaptic plasma membrane contribute significantly to our understanding of how cells control the consumption stage of the transport vesicle cycle. The clear involvement of lipids in this process was first described using a permeabilized neuroendocrine cell assay that reconstitutes dense core vesicle (DCV) fusion in two stages, preparation for fusion (priming) and a subsequent Ca^{2+}-dependent fusion step [50]. Priming depends on the action of a PITP and a PI 4-phosphate 5-kinase, whereas fusion requires a novel cytosolic neuron-specific factor termed 'calcium-activated

protein for secretion' (CAPS) [51,52]. The case for a fundamental role for CAPS in neurotransmission is amply made by the demonstration that CAPS insufficiencies in *Drosophila* result in deficits in DCV and synaptic vesicle release [53].

CAPS exhibits two membrane-binding domains, each essential for CAPS activity in neurotransmission. One is a central PH domain that binds acidic phospholipids, binds PIP_2 in a stereoselective manner, and is essential for CAPS binding to the plasma membrane but not to DCVs. The second is a carboxy-terminal domain required for CAPS binding to DCV membranes by an as yet unknown mechanism. This configuration suggests that CAPS acts as a tether that positions a dense core vesicle in apposition to the plasma membrane and facilitates Ca^{2+}-dependent fusion [54]. The presence of a PH domain on CAPS suggests a coupling between CAPS engagement with phosphoinositides generated during priming and fusion. Detailed analyses of the role of the CAPS PH domain in this coupling reveal that PH-domain mutations that inactivate CAPS as priming factor preferentially and unexpectedly compromise phosphatidylserine (PS) binding rather than PIP_2 binding [54]. These findings suggest a role for PS (perhaps in addition to PIP_2) in facilitating the long-range membrane–membrane interactions that precede DCV fusion.

The murine Munc13 proteins (like CAPS) belong to a conserved family of metazoan proteins that facilitate neurotransmission and are defined by the *Caenorhabditis elegans* protein Unc-13. Munc13s localize to the neuronal presynaptic zone and activate syntaxins so that the core vesicle fusion machinery can engage [55,56]. Genetic analyses in worms demonstrate a DAG requirement for Unc-13-dependent neurotransmission [57,58]. New evidence indicates that Munc13 represents the major DAG-activated component on the mammalian presynaptic plasma membrane [59•]. DAG binding is mediated via the Munc13 C1-domain, and this interaction effects Munc13 recruitment from the cytosol to the plasma membrane so that new sites for vesicle priming and fusion are created [59•].

Lipids and endocytosis

In yeast, the internalization step of endocytosis is sensitive to defects in the synthesis of sphingoid base [60]. Recent findings now indicate this sphingoid base requirement underlies a novel signal transduction pathway. This signaling cascade works through the action of two partially redundant -base-activated kinases (Pkh1p and Pkh2p) that phosphorylate yeast PKC, an important component of the yeast endocytic machinery [61]. Also, overproduction of dihydrosphingosine phosphate lyase in yeast compensates for defective endocytosis of the yeast v-SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) proteins Snc1p and Snc2p that are required for fusion of post-Golgi secretory vesicles to the plasma membrane [62]. In this case, however, the data suggest that modulation of sphingoid base metabolism results in altered sorting in the late stages of the secretory pathway so that some other protein replaces Snc1p/Snc2p as the operative v-SNARE of secretory vesicles.

The idea that vesicle coat proteins bind membranes and then deform them to initiate vesicle budding is a long-standing one. Structural data now suggest that members of the epsin family, accessory proteins that collaborate with clathrin and the AP2 adaptor complex in the budding of clathrin-coated vesicles, do just that. Epsins contain an epsin amino-terminal homology (ENTH) domain that constitutes a PIP_2-binding domain where the basic residues that form the phosphoinositide-binding site are distributed among several α helices. The net result is that PIP_2 binding induces a conformational change in the ENTH domain that effects surface bending of liposome membranes [63••]. Because epsin binds clathrin and the AP2 complex, which also binds PIP_2, an attractive model linking membrane bending to cargo recruitment via multivalent protein–lipid interactions presents itself.

Cholesterol is also suggested to act as a co-factor in a protein–lipid interaction-mediated membrane deformation pathway during the biogenesis of synaptic-like microvesicles from the neuroendocrine plasma membrane. Synaptic-like microvesicles (SLMVs) are cholesterol-rich structures, and their biogenesis is exquisitely sensitive to cholesterol deprivation. Synaptophysin, a major integral membrane component of these vesicles, is specifically labeled by a cholesterol photoprobe when it is uncaged in the context of an intact neuroendocrine cell [64]. Although there is no direct evidence that polymerization of synaptophysin–cholesterol complexes is sufficient to induce membrane bending, the interpretation of the data is a reasonable one that is amenable to experimental scrutiny.

A second mechanism for membrane deformation involves the enzymatic remodeling of lipids to effect shape changes that support vesicle budding. Endophilin, which exhibits a lyso-PtdOH acyltransferase activity, has been suggested to play such a role in the fission of vesicles from the Golgi complex [65,66]. *In vivo* studies confirm that endophilin is essential for clathrin-mediated endocytosis in living synapses [67••,68]. Analysis of endophilin-null mutants in *Drosophila* surprisingly demonstrates that such animals survive into the second or early third instar larval stage and that such larvae manage to sustain significant levels of neurotransmission during high frequency stimulation [67••]. The data suggest that 'kiss-and-run' fusion/fission mechanisms of neurotransmitter release account for the endophilin-independent regeneration of synaptic vesicles that sustains neurotransmission in these mutants.

Interestingly, dynamin insufficiencies result in failure to sustain high-frequency stimulation and indicate a role for dynamin in the 'kiss-and-run' vesicle cycle [69,70]. Because endophilin is dispensable for formation and fission of exocytotic vesicles and for the fission step of the 'kiss-and-run' cycle [67••], this raises the question of whether the lipid-remodeling activity of endophilin is directly relevant to fission of clathrin-coated vesicles in a physiological setting.

Conclusions and perspectives

As the lipid requirements for membrane trafficking are progressively unraveled, it is becoming abundantly clear that many lipid species play important and diverse roles in the exocytotic program. These functions are apparent at every stage of the secretory pathway and involve an impressive variety of lipids. In particular, the involvement of members of the more abundant lipid classes such as PS, PC and cholesterol in addition to minor and more specialized species, for example the phosphoinositides, in this process blurs classical distinctions between which lipids are considered signaling lipids and which are considered household lipids. Finally, emerging data only further emphasize the fact that an intimate interface must exist between activity of the secretory pathway and the regulation of lipid metabolic pathways that produce and consume lipids that regulate exocytotic functions. A large challenge for the future is elucidation of the mechanisms by which eukaryotic cells execute a temporal and spatial coordination of the essential interface between lipid metabolism and activity of the secretory pathway.

Acknowledgements

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