Lipid Sorting and Multivesicular Endosome Biogenesis

Christin Bissig and Jean Gruenberg

Biochemistry Department, University of Geneva, 1211 Geneva 4, Switzerland

Correspondence: jean.gruenberg@unige.ch

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Intracellular organelles, including endosomes, show differences not only in protein but also in lipid composition. It is becoming clear from the work of many laboratories that the mechanisms necessary to achieve such lipid segregation can operate at very different levels, including the membrane biophysical properties, the interactions with other lipids and proteins, and the turnover rates or distribution of metabolic enzymes. In turn, lipids can directly influence the organelle membrane properties by changing biophysical parameters and by recruiting partner effector proteins involved in protein sorting and membrane dynamics. In this review, we will discuss how lipids are sorted in endosomal membranes and how they impact on endosome functions.

It is now well established that membranes along the endocytic and secretory pathway show differences not only in protein but also in lipid composition. For example, lipid gradients exist along the biosynthetic pathway with increasing density of cholesterol and sphingolipids from the endoplasmic reticulum (ER) to the plasma membrane (Maxfield and van Meer 2010). Also, phosphoinositides show distributions restricted to relatively well-characterized membrane territories (Di Paolo and De Camilli 2006). Given the facts that lipids are small and contain little structural information when compared with proteins, that they can diffuse rapidly within membranes, and that membranes are connected by membrane flow during transport, it is not always obvious how different lipids are segregated from each other.

In this article, we will evoke different mechanisms that may contribute to the heterogeneous lipid composition of endocytic membranes, including physicochemical properties of the membrane, interactions with other proteins or lipids, and synthesis or degradation. In addition, it has also become apparent that peripheral membrane proteins often interact with membranes via diverse lipid-binding motifs, and thus that lipids directly contribute to the distribution of many peripheral membrane proteins. For example, phosphatidylinositol 3-phosphate (PI(3)P) is detected predominantly on early endosomes, where most characterized PI(3)P-binding proteins encoded by the human genome are found as well (Raiborg et al. 2013). We will also discuss how some lipids may regulate protein sorting and membrane transport within the endosomal system.

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ORGANIZATION OF THE ENDOSOMAL PATHWAY

Animal cells take up solutes, nutrients, ligands, and components of the plasma membrane via multiple endocytic pathways, which all merge in common early endosomes (Mayor and Pagano 2007; Doherty and McMahon 2009; Donaldson et al. 2013; Johannes et al. 2013; Kirchhausen et al. 2013; Merrifield and Kaksonen 2013). There, cargo can be sorted to different cellular destinations, including the plasma membrane, the *trans*-Golgi network (TGN), or late endosomes. Early endosomes contain tubular and cisternal elements, as well as vacuolar regions, where intralumenal vesicles (ILVs) of well-defined size, with a diameter of ~50 and ~30 nm in mammalian and yeast cells, respectively (Mari et al. 2008; Pons et al. 2008; Wemmer et al. 2011), are formed by budding of the limiting membrane away from the cytosol toward the endosome lumen (Fig. 1) (Gruenberg 2001; Huotari and Helenius 2011). This process is interesting from a mechanistic and topological point of view, because it occurs in a direction opposite to classical membrane budding leading to vesicle or tubule formation in both the secretory and endocytic pathways (Hurley et al. 2010; Henne et al. 2013). Eventually, these multivesicular regions mature or detach from early endosomes and become free multivesicular endosomes, often referred to as multivesicular bodies or endosomal carrier vesicles (MVBs/ECVs), which deliver their ILV cargo to late endosomes and lysosomes for degradation. Much like early endosomes, late endosomes are highly pleiomorphic in mammalian cells with tubulo-cisternal as well as complex multivesicular or multilamellar regions. Also, endosomes may well contain different types of ILVs, since not all ILV proteins and lipids are destined for lysosomes, as discussed below.

The endocytic pathway can be divided into two functional territories: the reutilization/recycling and the degradation territories that are connected by membrane transport (Gruenberg 2001; Huotari and Helenius 2011). The first territory defines the trafficking pathway between the plasma membrane and early endosomal elements and amounts to the most prominent intracellular trafficking routes in mammalian cells, which are primarily used by constitutively cycling, housekeeping receptors. This territory is kept well segregated from late endocytic compartments, where degradation occurs, and *trans*-territory traffic is ensured by ECV/MVB-mediated long distance, microtubule-dependent transport (Fig. 1).

PHOSPHOLIPID COMPOSITION OF ENDOSOMAL MEMBRANES

Lipids have the potential to generate 9000–100,000 different molecular species (Shevchenko and Simons 2010), and it has long been appreciated that lipid species are not distributed equally among cellular membranes. It is becoming apparent that this heterogeneity in lipid distribution may provide a general organizing principle for the distribution of membrane-associated proteins. In fact, it was recently proposed that the vacuolar apparatus be divided into two main membrane regions (Leventis and Grinstein 2010; Bigay and Antonny 2012). In the ER/*cis*-Golgi, loose packing of poorly charged monounsaturated lipids would favor interactions with cytosolic proteins that prefer lipid-packing defects, whereas in the *trans*-Golgi/plasma membrane/endocytic membranes, tight packing of saturated, negatively charged lipids would favor electrostatic interactions.

In addition to the general principles outlined above, some specialized lipids are also distributed in a nonstochastic manner along the endocytic pathway (Fig. 1). The plasma membrane contains relatively high amounts of cholesterol and glycosphingolipids, and some lipids are asymmetrically distributed between the two bilayer leaflets. Although glycosphingolipids are only localized to the outer leaflet, phosphatidylethanolamine (PE) and phosphatidylserine (PS) are only present in the inner leaflet of the plasma membrane (Devaux 1991; Emoto et al. 1996). It is generally believed that early endosomes share the same overall lipid composition as the plasma membrane, as they are both part of the same recycling territory. Like the plasma membrane, early endosomes are rich in cholesterol and PS (Gagescu et al. 2000; Mobius et al. 2003;

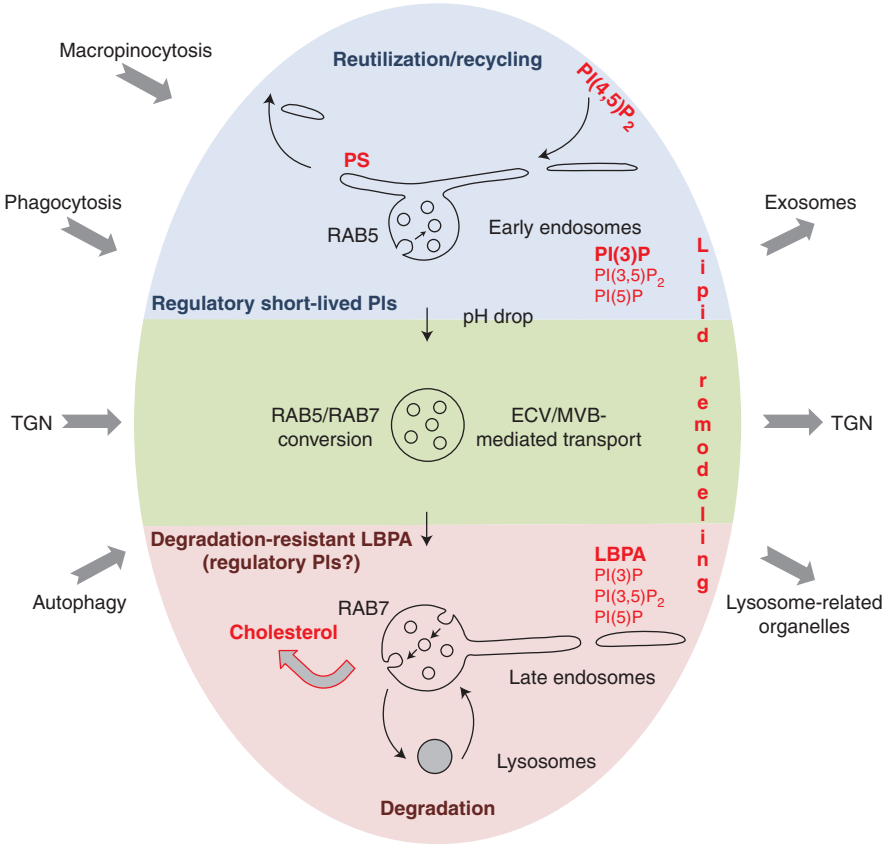


Figure 1. Organization of the endosomal pathway. In mammalian cells but perhaps not in yeast, the endocytic pathway can be divided into two functional territories, recycling/reutilization vs. degradation, which are linked by membrane transport. The plasma membrane and early endosomal elements belong to the recycling/reutilization territory through which large amounts of fluid or membrane are trafficked, ~30% of the cell volume and the surface area of the plasma membrane per hour (Steinman et al. 1976; Besterman and Low 1983; Steinman et al. 1983), or perhaps more (Howes et al. 2010a). In early endosomes, cargo is sorted for recycling/reutilization or degradation. In the latter case, transport to late endosomes occurs via endosomal carrier vesicles/multivesicular bodies (ECVs/MVBs), where RAB5-to-RAB7 conversion may occur (Rink et al. 2005). This transport step ensures that recycling and degradation pathways remain well segregated. Importantly, the reutilization/recycling and degradation compartments not only fulfill distinct functions but also show different protein and lipid compositions. Regulatory short-lived phosphoinositides mediate dynamics of endosomal elements in the reutilization/recycling pathway. Less is known about the role of regulatory phosphoinositides at late stages of the degradation pathway, where degradation-resistant LBPA (lysobisphosphatidic acid) membranes may mediate intraendosomal dynamics and cholesterol export. Importantly, the endosomal pathway also serves as an input or output for other membrane trafficking pathways, as indicated.

Leventis and Grinstein 2010; Fairn et al. 2011). However, the two compartments differ in their phosphoinositide composition, with $PI(4,5)P_2$ and $PI(3,4,5)P_3$ found at the plasma membrane and PI(3)P on early endosomes (Gillooly et al. 2000). PI(3)P is not restricted to the endosome-limiting membrane, where it is synthesized, but it also seems to be relatively abundant in ILVs of multivesicular endosomes early in the pathway (Gillooly et al. 2000; Mobius et al. 2003). Cholesterol may show a somewhat similar distribution as PI(3)P (Gillooly et al. 2000; Mobius et al. 2003). Late endosomes are rich in lysobisphosphatidic acid (LBPA) (or bis(monoacyl-

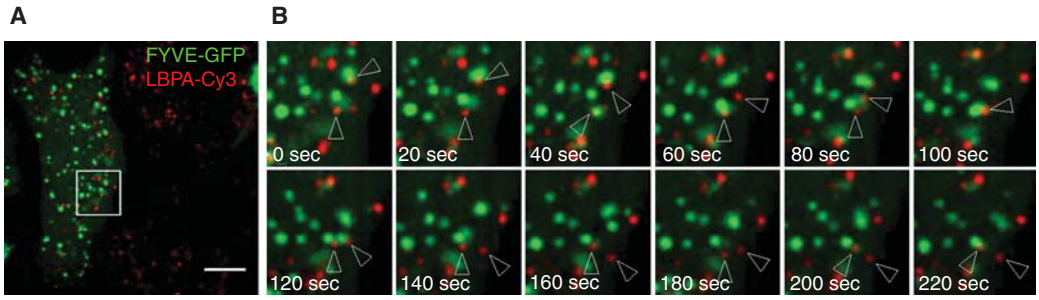


Figure 2. Dynamics of PI(3)P- and LBPA-containing endosomes in live cells. (*A*) HeLa cells were transfected with tandem FYVE-GFP and incubated overnight with mouse anti-LBPA and antimouse Cy3 antibodies. The concentration of anti-LBPA antibody (2.5 μg/mL in medium) and the levels of tandem FYVE-GFP expression were low to avoid possible interference with endosome dynamics. The cells were imaged in vivo and movies were taken at a frame rate of 0.6 sec by time-lapse confocal microscopy. Panel *A* shows in a still corresponding to the first frame that both cells contain endocytosed anti-LBPA antibodies but only the left cell expresses tandem FYVE-GFP. (*B*) Selected frames of the boxed magnified region. Arrowheads are showing movements of LBPA-positive endosomes. Scale bar, 10 μm. (From Bissig et al. 2012; reprinted, with permission, from Elsevier.)

glycero)phosphate, BMP), which is not detected elsewhere in the cell, including in early endosomes, and is abundant in ILVs (Kobayashi et al. 1998). Endosomes may contain more than one population of ILVs with differences in fate and composition. Indeed, LBPA is present in ILVs of late, but not early, endosomes, whereas PI(3)P shows the opposite distribution (Fig. 2) (Gillooly et al. 2000; Kobayashi et al. 2002; Chevallier et al. 2008). Furthermore, LBPA is not enriched in exosomes, which may correspond to ILVs secreted after multivesicular endosome fusion with the plasma membrane (Wubbolts et al. 2003; Laulagnier et al. 2004). Despite continuous membrane flow, the phospholipid composition of each compartment is maintained, indicating that cells evolved mechanisms to sort lipids within compartments.

LIPID CLUSTERING

Lipid Clustering by Physicochemical Properties In Vitro

Although it is easy to perceive that proteins can be sorted via specific signals encoded in their peptide sequences, the notion of lipid sorting is not clearly defined, and is a matter of debate. If lipids contribute to their own sorting and thus to their restricted localization, one must evoke mechanisms based on their physical and chemical properties, which may in turn affect the bilayer curvature and electrostatics, as well as lipid packing (Callan-Jones et al. 2011; Bigay and Antonny 2012). In vitro, some lipid species tend to segregate from others probably because of their different physicochemical properties. In addition, when compared with flat membranes, highly curved membranes accommodate different lipid species in vitro provided that these are close to phase separation, to compensate for membrane compression and bending stiffness (Roux et al. 2005; Sorre et al. 2009; Tian and Baumgart 2009). Lipid–lipid interactions play an important role in in vitro lipid segregation processes. In line with this, the raft hypothesis predicts that cholesterol, sphingolipids, and proteins, upon demixing from the surrounding membrane components by phase separation, form dynamic nanoscale assemblies, referred to as lipid rafts, which may coalesce into larger platforms involved in signaling and trafficking (Lingwood and Simons 2010). In fact, it has been recently proposed that liquid phase separation may be of wider importance, as one of the underlying principles of cytoplasmic and nuclear compartments (Hyman and Simons 2012).

Lipid Clustering In Vivo

At the cell surface, both simian virus 40 and Shiga toxin can cluster their glycosphingolipid re-

ceptors and thus impose a geometry on the lipids, which induces membrane tubulation and leads to lipid sorting into these invaginations (Romer et al. 2007; Ewers et al. 2010). Owing to its lipid and actin dependence, this pathway is reminiscent of the CLIC-GEEC pathway that mediates internalization of glycosylphosphatidylinositol (GPI)-anchored proteins (Howes et al. 2010b). However, much less is known about protein-independent clustering of lipids.

One of the major limitations in studying lipid organization in membranes in vivo is that lipid nanostructures are very difficult to visualize, because of their small size and highly dynamic nature (Prior et al. 2003; Sharma et al. 2004). High-resolution mapping by electron microscopy now revealed that PS is not homogenously distributed within the plasma membrane, but is localized to nanoclusters, which are associated with cholesterol and sphingomyelin-rich caveolae (Fairn et al. 2011; Kay et al. 2012). Interestingly, cholesterol and sphingomyelin are also found together with PS in early and recycling endosomes (Gagescu et al. 2000; Mobius et al. 2003; Mondal et al. 2009). Thus, one may speculate that PS preferentially copartitions with cholesterol-rich membranes in vivo, which are in turn targeted to the recycling pathway. If specialized lipids tend to assemble in nanodomains in vivo, these are unlikely to result strictly from the physicochemical properties of the lipids, but presumably arise from a combination of lipid–lipid, protein–protein, and lipid–protein interactions. Indeed, integral and peripheral proteins may influence—but also show preferences for—physical or chemical membrane properties, and thus may be preferentially sorted into curved versus flat or tightly versus loosely packed membranes (Callan-Jones et al. 2011; Bigay and Antonny 2012).

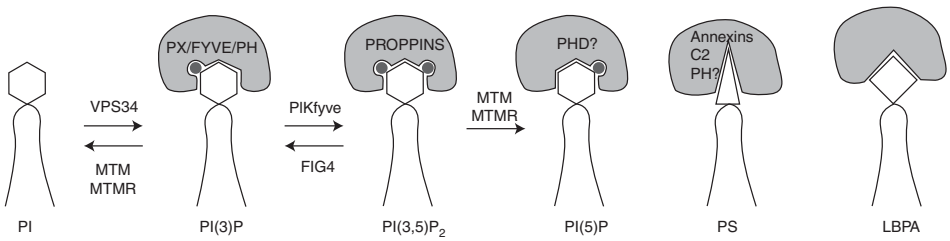
RESTRICTED LIPID LOCALIZATION VIA SPATIALLY AND TEMPORALLY CONTROLLED SYNTHESIS AND TURNOVER: SHORT-LIVED PHOSPHOINOSITIDES

Lipid restriction to distinct compartments might also be achieved by temporal and spatial regulation of lipid synthesis and turnover (Fig. 3A). Such a mechanism is well established for short-lived phosphoinositides that are rapidly turned over by kinases and phosphatases, but might also apply for other lipids (see below).

PI(3)P is generated on early endosomes mostly by the action of the class III PI3-kinase, VPS34, which phosphorylates the D-3 position of the inositol ring of PI (Schu et al. 1993; Shin et al. 2005) and is turned over by 3-phosphatases, such as mammalian myotubularin (MTM)- and myotubularin-related (MTMR) (Clague and Lorenzo 2005). Generation of PI(3)P on early endosomes is directly controlled by the small GTPase RAB5, a master regulator of early endosomal dynamics, because VPS34 is itself an RAB5 effector. In turn, several RAB5 effectors, such as EEA1 (early endosome antigen 1) and rabenosin-5, contain themselves a PI(3)P-binding FYVE (Fab1p, YOTB, Vac1p, EEA1) domain that is required for endosomal targeting. Thus, RAB5 stimulates PI(3)P synthesis in a positive-feedback loop (Zerial and McBride 2001). The 3-phosphatase MTM1 was shown to directly associate with VPS34 and is thus also localized to early endosomes (Cao et al. 2007). Although it remains unclear whether VPS34 and MTM1 are simultaneously active, their concomitant presence on early endosomes points toward a local and rapid mechanism of PI(3)P turnover.

Furthermore, PI(3)P can be metabolized to $PI(3,5)P_2$ by the PI(3)P 5-kinases Fab1p in yeast (Odorizzi et al. 1998) and PIKfyve in mammalian cells (Sbrissa et al. 2002b). The localization of $PI(3,5)P_2$ within the endocytic pathway is still unclear, because direct intracellular visualization of the lipid has been extremely difficult until now, as all $PI(3,5)P_2$-binding PROPPINs (β propellers that bind phosphoinositides) also interact with PI(3)P (Dove et al. 2004; Jeffries et al. 2004; Stromhaug et al. 2004; Baskaran et al. 2012). Furthermore, $PI(3,5)P_2$ localization is additionally complicated by the fact that it is a very minor lipid in nonstimulated cells, although its synthesis can be induced by osmotic stress (Cooke et al. 1998). The kinase PIKfyve/Fab1p, which synthesizes $PI(3,5)P_2$, contains a PI(3)P-binding FYVE domain that presumably recruits the protein to early endosomes (Cabezas et al. 2006; Ikonomov et al. 2006; Rutherford et al. 2006), in which the substrate PI(3)P is also

A



B

Lipid	PI(3)P						$PI(3,5)P_2$	PI(5)P	PS	Cholesterol	LBPA
Lipid localization	EE/MVB[a]						EE-to-LE[h]	?	PM/EE/RE[k]	Endocytic pathway[m]	LE[o]
Protein	RAB effectors[b]	SNX1 SNX2[c]	SNX3[d]	HRS[e]	ALFY[f]	PIKfyve[g]	Atg18 Atg21[i]	?	EVT2[l]	NPC1 NPC2[n]	ALIX HSP70[p]
Lipid-binding domain	FYVE PX	PX BAR	PX	FYVE	FYVE	FYVE	PROPPIN	?	PH		
Protein function	Docking/ fusion Motility	Retrograde transport	ILV formation Retrograde transport Iron regulation	ILV formation Receptor sorting	Autophagy	$PI(3,5)P_2$ synthesis	Autophagy	Receptor degradation /sorting[j]	Retrograde transport	Endosome dynamics	ILV formation and fission Lysosome stabilization

Figure 3. Phospholipid-binding domains and proteins involved in endosomal dynamics. (*A*) A schematic illustration of key endosomal lipids and their phospholipid-binding domains is shown, including kinases and phosphatases involved in endosomal phosphoinositide generation and turnover. However, other pathways may also exist for the generation of PI(5)P. Enzymes involved in the metabolism of phosphatidylserine (PS), which is synthesized in the ER like most phospholipids, are not shown. The enzymes involved in LBPA synthesis and degradation are not known. (*B*) Key endosomal lipids and their localization (light gray) are shown with the corresponding phospholipid-binding proteins and their endosomal functions (dark gray). [a]Mobius et al. 2003; [b]Simonsen et al. 1998; McBride et al. 1999; Pankiv et al. 2010; [c]Carlton et al. 2004; Bonifacino and Hurley 2008; [d]Pons et al. 2008; Harterink et al. 2011; Chen et al. 2013; [e]Lloyd et al. 2002; Bache et al. 2003; Pons et al. 2008; [f]Simonsen et al. 2004; Filimonenko et al. 2010; [g]Sbrissa et al. 2002a; [h]Cabezas et al. 2006; Ikonomov et al. 2006; Rutherford et al. 2006; [i]see Tooze and Elazar 2013; and references in Mayinger 2012; [j]Ramel et al. 2011; Zolov et al. 2012; Oppelt et al. 2013; [k]Gagescu et al. 2000; Leventis and Grinstein 2010; Fairn et al. 2011; [l]Uchida et al. 2011; [m]Mobius et al. 2003; Ikonen 2008; Maxfield and van Meer 2010; [n]Vanier and Millat 2003; Ohgami et al. 2004; Xu et al. 2007; Kwon et al. 2009; [o]Kobayashi et al. 1999, 2002; [p]Matsuo et al. 2004; Kirkegaard et al. 2010; Bissig et al. 2013.

found. It is thus likely that $PI(3,5)P_2$ synthesis begins in early endosomes, and may continue at later stages of the pathway. $PI(3,5)P_2$ turnover is mediated by the 3-phosphatases of the MTM/MTMR family and the 5-phosphatase factor-induced gene 4 protein (Fig. 4), which is found in a complex with PIKfyve (Duex et al. 2006), showing that synthesis and turnover are tightly coupled. The activity of MTM/MTMR phosphatase may also generate PI(5)P (Zolov et al. 2012; Oppelt et al. 2013), a minor lipid that can be increased by various stimuli and might also play a role in lysosomal targeting (Fig. 3) (Ramel et al. 2011).

LIPIDS AS ORGANIZING PRINCIPLES OF PERIPHERAL MEMBRANE PROTEINS

Many proteins involved in the regulation of endosomal dynamics contain phospholipid-bind-

ing domains (Fig. 3B). These phospholipid-binding domains are typically present in proteins that interact transiently with membranes and thus show relatively low binding affinity toward their target lipids to ensure rapid association-dissociation kinetics. These domains interact with the membrane either via specific recognition of rare lipids, such as phosphoinositides, or via recognition of physicochemical properties of the bilayer, such as curvature, lipid packing, or electrostatics (Bigay and Antonny 2012). Additionally, phospholipid-binding domains are often accompanied by other lipid- or protein-binding motifs, thus allowing for coincidence detection of multiple factors to stabilize proper membrane localization (Moravcevic et al. 2012). Thus, lipids act as organizing principles for membrane-associated proteins and their functions.

PI(3)P-Controlled Membrane Dynamics, Sorting, and Transport in Endosomes

PI(3)P regulates most, if not all, key early endosome functions, including membrane dynamics, lysosome targeting, and retrograde transport (Figs. 1 and 3B), by the recruitment of effector proteins that interact with PI(3)P via specific lipid-binding domains, such as the FYVE domain and the PX (phox) domain (Lemmon 2008). The role of PI(3)P in early endosome membrane dynamics is highlighted by the fact that many effectors of the small GTPase RAB5 are themselves PI(3)P-binding proteins, including the FYVE domain-containing tethering factor EEA1, which interacts with SNARE proteins in membrane docking/fusion (Simonsen et al. 1998; McBride et al. 1999). The role of RAB proteins, including the RAB5-to-RAB7 conversion that accompanies ECV/MVB-mediated transport from early-to-late endosomes (Fig. 1) is discussed elsewhere in the collection (Pfeffer and Wandinger-Ness 2013). PI(3)P also plays a direct role in endosome mobility by recruiting the kinesin-3, KIF16B, which contains a PI(3)P-binding PX domain (Hoepfner et al. 2005). Similarly, the plus end-directed transport of autophagic vesicles, which fuse with late endocytic elements, is promoted by the PI(3)P effector FYCO1 (FYVE and coiled-coil domain-containing protein 1) (Pankiv et al. 2010), in line with the fact that PI(3)P is required for autophagosome biogenesis via recruitment of PI(3)P effectors (Simonsen et al. 2004; Filimonenko et al. 2010; Noda et al. 2010; Tooze et al 2013).

In addition, PI(3)P also initiates the molecular sequence of events leading to the down-regulation of ubiquitinated cargo proteins in lysosomes, including activated signaling receptors. Indeed, inhibition of PI(3)P synthesis with wortmannin, a drug that inhibits PI3-kinases, impairs ILV formation (Fernandez-Borja et al. 1999), and traps signaling receptors in early endosomes, thus inhibiting their degradation (Petiot et al. 2003). The effects of wortmannin reflect the role of PI(3)P in the recruitment of ESCRT complexes that mediate sorting of ubiquitinated cargo proteins into ILVs, and in particular, the recruitment of the ESCRT-0 subunit HRS, a PI(3)P-binding protein that contains a FYVE motif, and initiates the sorting sequence. Depletion of HRS in mammalian cells (Bache et al. 2003; Pons et al. 2008) or mutagenesis in flies (Lloyd et al. 2002), decreases ILV formation and impairs signaling receptor degradation. However, the time course and extent of epidermal growth factor (EGF) transcriptional response do not appear to be affected by depletion of HRS or other ESCRT subunits, suggesting that the EGF response is set in motion primarily by active receptors present at the cell surface or perhaps also in endosomal elements of the reutilization/recycling territory (Fig. 1) (Brankatschk et al. 2012; Sousa et al. 2012). In addition, we find that ILV formation, but not EGF receptor degradation, is inhibited by depletion of SNX3, another PI(3)P-binding protein containing a PX motif (Pons et al. 2008, 2012), indicating that lysosomal targeting can be uncoupled from ILV formation, in agreement with previous observations (Mayran et al. 2003; Morel et al. 2009). Moreover, SNX3 in complex with VPS26-VPS29-VPS35 may also regulate very selectively the endosome-to-TGN transport of Wntless (Harterink et al. 2011). Recent evidence indicates that SNX3 may also control iron assimilation and transferrin receptor recycling (Chen et al. 2013). The mechanism by which SNX3

regulates membrane dynamics, Wntless transport, iron assimilation, and ILV formation, is still elusive. Remarkably, SNX3 does not contain any structural features other than the PX domain (Xu et al. 2001), and it remains to be investigated whether SNX3 directly induces membrane invaginations or whether it recruits other proteins, for example, ESCRT subunits.

Regulatory Short-Lived Phosphoinositides in Transport to/from Endosomes

PI(3)P-binding proteins also regulate retromer-dependent endosome-to-TGN transport. Retrograde transport and the retromer complex are discussed in detail elsewhere in this collection (Burd and Cullen 2013). The classical retromer complex is built of two subcomplexes: the membrane-binding SNX-BAR heterodimer and the cargo selection VPS26-VPS29-VPS35 subcomplex (Bonifacino and Hurley 2008; Cullen and Korswagen 2012). The SNX-BAR subcomplex, which is formed by a SNX heterodimer (SNX1 or SNX2 with either SNX5, SNX6, or SNX32), mediates retromer recruitment to early endosomes via its PI(3)P-binding PX domain and its curvature-sensing BAR domain (Carlton et al. 2004). As mentioned, SNX3 in complex with VPS26-VPS29-VPS35 might also regulate Wntless retrograde transport (Harterink et al. 2011).

PI(3)P is also a substrate for the generation of PI(3,5)P_2, which in turn can be metabolized to PI(5)P (Fig. 3A) (Zolov et al. 2012; Oppelt et al. 2013). It is very likely that PI(3,5)P_2 and PI(5)P, together with their effector proteins, also play active roles in endosomal dynamics. However, we still know relatively little about their exact functions. In yeast PI(3,5)P_2-binding PROPPIN proteins, such as Atg18 and Atg21, have been implicated in autophagosome maturation and vacuole homeostasis (Mayinger 2012; Tooze et al. 2013). Further roles of PI(3,5)P_2 in animal cells seem to be the activation of mucolipin transient receptor potential ion channel (Dong et al. 2010), regulation of autophagy in the nervous system (Ferguson et al. 2010), and cardiac contractility via activation of the ryanodine receptor (Touchberry et al. 2010). The role of PI(5)P is much less clear, but one can speculate that it is involved in lysosomal targeting of signaling receptors (Ramel et al. 2011). Altogether, these data indicate that phosphoinositides act as a fundamental organizing principle in the endocytic pathway (Fig. 1).

RESTRICTED LIPID LOCALIZATION VIA SPATIALLY CONTROLLED SYNTHESIS AND TURNOVER: LONG-LIVED LBPA

In late endosomes, LBPA makes up ~15 mole % of total phospholipids. The main LBPA isoform is 2,2′-dioleoyl LBPA, which accounts for >80% of total LBPA in baby hamster kidney cells (BHK) (Kobayashi et al. 1998, 2002) and seems to be the major biologically active isoform (Matsuo et al. 2004; Chevallier et al. 2008). 2,2′-LBPA is thermodynamically unstable, as the fatty acid chains can migrate to form 3,3′-LBPA (Fig. 4) (Chevallier et al. 2000; Kobayashi et al. 2002). The position of LBPA fatty acyl chains is expected to have dramatic effects on the overall structure of the lipid and is thus likely to modulate its biological functions. Quantum mechanical simulations of molecular electronic energies of 2,2′- and 3,3′-LBPA indeed suggest that the two isomers show significantly different shapes (Goursot et al. 2010).

Phospholipids of the vacuolar apparatus are synthesized in the early biosynthetic pathway and then redistribute throughout organellar membranes primarily by membrane flow, with some being unevenly distributed along trafficking pathways, as mentioned. It is thus surprising that LBPA, with its characteristic phospholipidic backbone (Fig. 4), is only found in late endosomal membranes, and is not detected elsewhere. This strongly argues for its localized synthesis in the late endosomal compartment from a phospholipidic precursor. Indeed, it has been shown that two polyglycerophospholipids, phosphatidylglycerol (PG), a structural isomer of LBPA and cardiolipin, can be converted to LBPA in in vivo systems (Poorthuis and Hostetler 1975; Somerharju and Renkonen 1980; Amidon et al. 1995). However, it was also shown that only PG, but not cardiolipin, serves as a possible precursor for de novo LBPA biosynthesis in

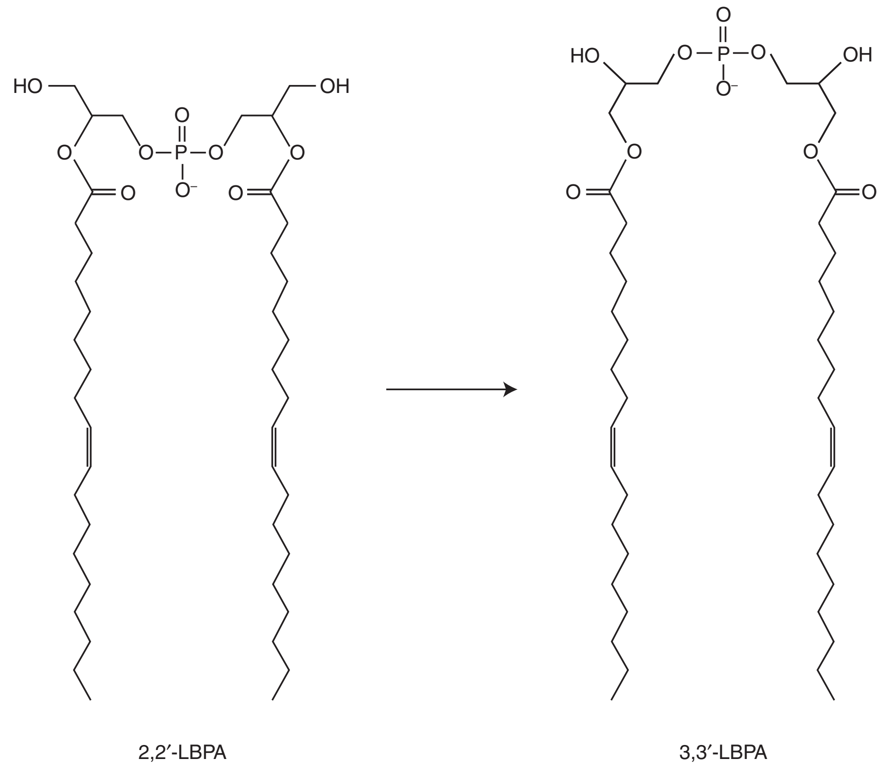


Figure 4. Chemical structures of the 2,2′- and 3,3′-dioleoyl LBPA isoforms. The major isoform present in late endosomes of BHK cells is 2,2′-dioleoyl LBPA. This isoform is thermodynamically unstable and the fatty acid chains can migrate to the 3,3′ positions of the glycerol backbone to form the 3,3′-LBPA isoform.

vivo (Hullin-Matsuda et al. 2007). Strikingly these studies also show that despite decreased de novo LBPA synthesis in cells deficient for PG synthesis, the total LBPA amount remains unchanged, indicating that some other biosynthetic pathway must exist. Moreover, PG, like cardiolipin, is primarily found in mitochondria suggesting that these lipids may be delivered to late endocytic compartments by autophagy (Rubinsztein et al. 2012), to serve as precursors in LBPA biosynthesis. Finally, LBPA is a poor substrate for lipases and phospholipases, which has led to the notion that it may be the stereoisomer of other naturally occurring phospholipids (Brotherus et al. 1974; Joutti 1979; Thornburg et al. 1991; Chevallier et al. 2000), as was recently shown (Tan et al. 2012). This notion is consistent with observations that LBPA facilitates the degradation of glycosphingolipids in vitro by enhancing the lipid-extraction capacity of saposins (Kolter and Sandhoff 2010). LBPA may also facilitate HSP70 entry into lysosomes and lysosome stabilization (Kirkegaard et al. 2010).

The ESCRT-associated cytosolic protein ALIX is recruited to endosomes via interactions with LBPA (Matsuo et al. 2004; Bissig et al. 2013) indicating that the lipid can be found on the cytoplasmic leaflet of the endosome-limiting membrane—and yet it is presumably synthesized in the endosome lumen from a precursor lipid, where it can be accessed by endocytosed antibodies (Kobayashi et al. 1998, 1999). Hence, if LBPA is present in both leaflets of the late endosomal-limiting membrane and if it is synthesized in late endosomes, it needs to be translocated to the cytoplasmic leaflet. Because of its low pKa, LBPA is expected to remain negatively charged at the late endosomal pH of 5.5 (Wilkening et al. 2000). However, LBPA self-assembly may cause partial protonation of proximal LBPA

phosphate groups. In this case, the protonated LBPA molecule could undergo spontaneous transbilayer redistribution. Alternatively, LBPA may be translocated across the bilayer by some as-yet-unidentified flippase. Finally, late endosomes, in contrast to ECVs/MVBs, contain complex and often poorly defined intralumenal protein–lipid systems—see, for example, Kobayashi et al. (2002) and Matsuo et al. (2004)—which may in part reflect nonbilayer structures in dynamic equilibrium with lumenal membranes, a situation that may favor LBPA redistribution across bilayers.

LIPID-MEDIATED ENDOSOMAL DYNAMICS

It is well established that endosomal membranes are endowed with the capacity to become deformed in two opposite directions: toward the cytosol much like at other membrane transport steps during tubule or vacuole biogenesis, and toward the endosome lumen during ILV biogenesis. These two membrane deformation processes must be tightly coordinated to maintain the homeostatic membrane balance. The molecular mechanisms are not well understood, although much progress has been made in the characterization of each process individually. Nascent tubules of recycling endosomes selectively incorporate the Man6P receptor sorted via the retromer complex, which is recruited onto membranes by the dual capacity of sorting nexins to interact with both PI(3)P via the PX domain and curved membranes via the curvature-sensing BAR domain (Bonifacino and Hurley 2008). Retromer recruitment and tubule biogenesis may be coordinated by RAB5-to-RAB7 conversion (Rojas et al. 2008; Seaman et al. 2009; Balderhaar et al. 2010; Liu et al. 2012; van Weering et al. 2012), and recent lines of evidence indicate that this process may also involve the actin cytoskeleton (Seaman 2012). During ILV formation, the sorting of ubiquitinated cargo proteins is achieved by ESCRT complexes, which may also be involved in the membrane deformation and scission processes (Wollert and Hurley 2010; Henne et al. 2012). However, in addition to proteins, it has long been thought that lipids also play an important role in membrane deformation; in particular, during the formation of intralumenal membranes (Mukherjee et al. 1999; Babst 2011).

It has been shown that LBPA has the intrinsic capacity to deform membranes in vitro (Matsuo et al. 2004), a situation that probably reflects ILV formation within late endosomes in vivo. However, only the 2,2′-LBPA isoform, but not 3,3′-LBPA, triggers vesicle formation within acidic liposomes, in a process that is controlled by its partner, the ESCRT-associated protein ALIX (Matsuo et al. 2004; Frederick et al. 2010). ALIX specificity toward LBPA is conferred by a flexible loop in the Bro1 domain that undergoes a conformational change upon membrane association and requires a calcium atom bound near the lipid interaction site (Bissig et al. 2013). ALIX also regulates the formation of ILVs within acidic late endosomes in vitro (Falguieres et al. 2008). The regulatory function of ALIX and LBPA in ILV formation is further supported by observations that ALIX knockdown reduces LBPA and the number of ILVs (Matsuo et al. 2004; Chevallier et al. 2008), and that both can be restored in this knockdown background by the addition of 2,2′-LBPA (Chevallier et al. 2008). Thus, LBPA molecules that appear on the endosome-limiting membrane may be reincorporated into nascent ILVs, a mechanism that may explain the restricted distribution of LBPA to multivesicular elements of late endosomes. Typically, LBPA is not detected in the dynamic, cisternal, or tubular regions of late endosomes, which contain the PX-containing protein SNX16 (Brankatschk et al. 2011). This may suggest that PI(3)P is also present in late endosomes or that SNX16 is stabilized on late endosomal membranes by other protein–lipid or protein–protein interactions. Importantly, interfering with LBPA (Luyet et al. 2008) or ALIX (Schmidt et al. 2004; Cabezas et al. 2005; Bowers et al. 2006; Doyotte et al. 2008; Luyet et al. 2008) does not affect the trafficking of EGF receptor to lysosomes, nor EGF receptor down-regulation, despite the fact that ALIX interacts with the two ESCRT subunits TSG101 and CHMP4 in mammalian cells. ALIX, however, may be involved in the ubiquitin-independent, ESCRT-III-dependent sorting of the GPCR PAR1 (Dores et al. 2012).

ENDOSOMAL TRAFFICKING OF CHOLESTEROL

Cholesterol is abundant in all post-Golgi membranes, together with sphingolipids, and their levels increase along the biosynthetic pathway, which presumably impacts on the length of protein transmembrane domains by changing membrane thickness (Sharpe et al. 2010). In the endocytic pathway, cholesterol is thought to localize primarily to recycling endosomes and multivesicular endosomes, where it is also abundant in ILVs (Mobius et al. 2003). In contrast, lysosomes are known to contain little cholesterol. Endosomes acquire cholesterol as a membrane component via trafficking routes, or as cholesteryl esters by receptor-mediated endocytosis of low-density lipoprotein (LDL)—the latter pathway constitutes the major cellular cholesterol source in most cell types (Brown and Goldstein 1976). In late endosomes, acid lipase hydrolyses cholesteryl esters and the resulting free cholesterol partitions into neighboring membranes. However, the mechanism of cholesterol export from endosomes to other cellular destinations is still a matter of debate (Ikonen 2008; Maxfield and van Meer 2010).

LDL-derived cholesterol and glycosphingolipids accumulate in late endocytic compartments of patients with Niemann-Pick type C (NPC) disease (Vanier and Millat 2003). This disease leads to progressive neurodegeneration and infantile death and is caused by mutations in NPC-1 or NPC-2 proteins (Miller and Bose 2011). NPC-1 is a 13-transmembrane-spanning glycoprotein found in the limiting membrane of late endosomes, whereas NPC-2 is a soluble protein of the late endosomal lumen. Biochemical and structural studies have shown that both proteins bind sterols (Ohgami et al. 2004; Xu et al. 2007; Kwon et al. 2009) and it has been proposed that the lumenal protein NPC-2 extracts cholesterol from ILVs and transports it to NPC-1, which mediates insertion into the limiting membrane (Infante et al. 2008). It has also been proposed that sphingosine acts as an initiating factor in the NPC pathogenesis by altering calcium homeostasis (Lloyd-Evans et al. 2008). Several lines of evidence indicate that the late endosomal lipid LBPA and its partner protein ALIX play a role in cholesterol export (Kobayashi et al. 1999; Le Blanc et al. 2005; Chevallier et al. 2008). After de-esterification, free cholesterol is likely to partition preferentially into ILV membranes as they are highly abundant, where it might readily flip-flop across the bilayer (Parisio et al. 2012). ALIX- and LBPA-dependent back fusion of ILVs with the limiting membrane may then control cholesterol export from endosomes.

LIPID-DEPENDENT EXPORT FROM ENDOSOMES

The notion that ILV back fusion may contribute to ensure cholesterol export from endosomes is further supported by observations that proteins and lipids present in late endosome intralumenal membranes are presumably not all destined for degradation in lysosomes, including LBPA itself and tetraspanins (Escola et al. 1998). Also molecules in transit through this compartment, including the Man6P receptor and the tetraspanin CD63 in endothelial cells, can escape delivery to lysosomes (Kobayashi et al. 1998, 2000). In addition, some ILVs with their protein and lipid cargo may be released as exosomes upon direct fusion of multivesicular endosomes with the plasma membranes, at least in some specialized cell types (Raposo and Stoorvogel 2013). This pathway is reminiscent of the secretory functions of lysosome-related organelles (Raposo et al. 2007), but it cannot be excluded that some multivesicular endosome population transiently acquires the capacity to fuse with the plasma membrane (Murk et al. 2002; Johnstone 2006). During melanosome formation, cargo sorting was recently shown to depend on CD63 but not ESCRTs (van Niel et al. 2011), whereas the biogenesis of exosomes may (Gibbings et al. 2009; Baietti et al. 2012) or may not (Trajkovic et al. 2008) depend on ALIX and ESCRTs. Moreover, vesicular stomatitis virus (VSV) (Le Blanc et al. 2005; Luyet et al. 2008; Bissig et al. 2013), Lassa virus, and lymphocytic choriomeningitis virus (Pasqual et al. 2011) hijack the endocytic pathway to gain entry into their host cells in a process that depends on LBPA, ALIX, and ESCRTs, as does the anthrax toxin lethal factor

(Abrami et al. 2004). Both VSV nucleocapsid and anthrax toxin lethal factor may first be delivered into ILV lumen early in the pathway and then released into the cytoplasm by ILV back fusion with the endosome-limiting membrane (Abrami et al. 2004; Le Blanc et al. 2005; Luyet et al. 2008), a process presumably similar to that followed by cholesterol during export from endosomes.

Given the possible role of LBPA in ILV formation and back fusion, we speculate that LBPA forms microdomains or hot spots on the endosomal-limiting membrane, and thereby controls ILV dynamics via kiss-and-run fusion and fission cycles. Hence, it is attractive to conclude that ILVs function as sorting devices in the endosomal system.

CONCLUDING REMARKS AND FUTURE CHALLENGES

The significance of the vast diversity of lipid species is poorly understood (Shevchenko and Simons 2010). This diversity primarily is owing to the enormous repertoire of possible acyl chain positions, saturation, and length, as well as linkages, including ether and amide in addition to the common ester bond (Wenk 2005). In particular, for LBPA, differences in amounts and acyl chain composition, including length and saturation state, are observed in different cell types (e.g., Luquain et al. 2000; Kobayashi et al. 2002), and under pathological conditions (Vanier and Millat 2003; Meikle et al. 2008), suggesting that LBPA functions can be tuned not only by its abundance but also by changes in its composition. A future challenge will be to determine how cellular functions are regulated by the molecular structure of individual lipids as well as by the membrane lipid composition.

Although proteins can be manipulated by mutagenesis, including even at endogenous levels in genome-edited cells (Doyon et al. 2011), similar experiments are difficult or impossible when dealing with lipids. Changing the molecular structure of a lipid, in contrast to proteins, may affect the lipid architecture and its physicochemical properties so profoundly that it is no longer the same molecular species and thus shows different functional properties. And interfering with metabolic enzymes often results in compensatory changes in overall lipid homeostasis (Guan et al. 2009) or leads to the accumulation of a lipid intermediate with different properties, rendering such approaches difficult to interpret. A powerful alternative for interfering with the functions of lipids, and, in particular, of phosphoinositides, is the expression of lipid-specific reporter proteins (Lemmon 2008; Mayinger 2012). Yet, the selective manipulation of individual lipids in vivo remains a major challenge. In specific cases, the exogenous addition of lipids has proven to be useful. For example, the addition of aminophospholipid increases endocytosis in living cells (Farge et al. 1999) and adding the biologically active isoform, but not other isoforms, of LBPA reduces cholesterol overload in NPC cells (Chevallier et al. 2008). Finally, a promising approach has been elaborated by Carsten Schultz and colleagues that developed very elegant strategies to introduce membrane-permeant versions of phosphoinositides into cells so that the lipid is released upon cleavage by cytosolic esterases (Laketa et al. 2009; Mentel et al. 2011). Such strategies, together with the development of novel chemical biology tools, offer promising new ways to study the function of membrane lipids (Riezman and Johnsson 2011; Wymann and Schultz 2012).

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Christin Bissig and Jean Gruenberg

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