ELSEVIER

Lipids in endocytic membrane transport and sorting

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Protein complexes associated to specific membrane lipids and protein–lipid domains contribute to regulate protein sorting and membrane dynamics in the endocytic pathway. It is also becoming apparent that different lipid territories are distributed along the pathway, and that some lipids segregate into specialised microdomains.

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Abbreviations

AP2	clathrin-associated adaptor complex 2
ECV	endosomal carrier vesicle
EGFR	epidermal growth factor receptor
ESCRT	endosomal sorting complex required for transport
FYVE	Fab1p/YOTB/Vac1p/EEA1
LBPA	lysobisphosphatidic acid/bis(monoacylglycerol) phosphate
MVB	multivesicular body
NPC	Niemann–Pick type C
PH	pleckstrin homology
PI3P	phosphatidylinositol 3-phosphate
PI(3,5)P2	phosphatidylinositol 3,5-bisphosphate
PI(4,5)P2	phosphatidylinositol 4,5-bisphosphate
PX	PHOX homology
SNX	sorting nexin
TGN	*trans*-Golgi network

Introduction

Lipids provide the physical support of organelle membranes, acting as a barrier for water-soluble molecules and as a solvent for the hydrophobic domains of membrane proteins. By contributing to the intrinsic properties of membranes, such as thickness, asymmetry and curvature, lipids can potentially regulate protein movement and distribution. In addition, different, not mutually exclusive, roles have also been assigned to lipids. Many cytosolic proteins interact with membranes by binding not only to proteins but also to lipids, often through multiple protein–lipid and protein–protein interactions. Such interactions are not easily studied, however, and it should be emphasised that physiologically relevant parameters, for example kinetic constants, are rarely known. Evidence is also accumulating that some short- and long-lived lipids have a restricted distribution in the plane of the bilayer, thereby forming transient or more stable microdomains.

Here, I will discuss the roles of protein–lipid microdomains and other lipid-based molecular assemblies in endocytic membranes.

Clathrin-coated vesicles and membrane curvature

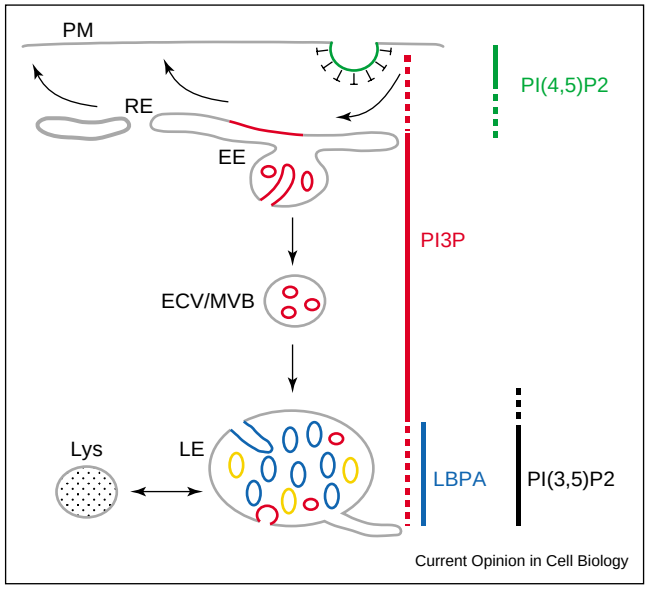
Receptors and other proteins, lipids and solutes are endocytosed by clathrin-coated pits and vesicles and then appear in early endosomes [1], but other routes of entry also exist, including phagocytosis, macropinocytosis and other less well-characterised pathways [2,3]. Clathrin-coated pit formation involves a network of interactions between clathrin, the adaptor complex AP2 and associated proteins [4]. This process, which is spatially and temporally regulated [5], also depends on PI(4,5)P2 (phosphatidylinositol 4,5-bisphosphate) actin and actin regulators [6] — a mechanism that might bear some similitude to COP-coated vesicle formation on Golgi membranes [7].

In addition to the possible role of transbilayer phospholipid translocation [8], protein–lipid interactions underlie the processes that generate membrane curvature during clathrin-coated pit formation. Four proteins, which interact with each other, generate curvature in bilayers and tubulate artificial membranes, including the GTPase dynamin, involved in vesicle fission, endophilin and amphiphysin [9]. Amphiphysin 2, together with caveolin-3 and presumably other factors, might in fact cause membrane deformation during T-tubule biogenesis in muscle [10]. In addition, epsin, which binds clathrin, Eps15 and AP2 (clathrin-associated adaptor complex 2), also generates curvature and tubulates bilayers — presumably by inserting a helix into the inner leaflet of the bilayer — and the activity depends on PI(4,5)P2 [11••]. Because epsin also facilitates clathrin polymerisation, and might be required for AP2 targeting, it has been speculated that membrane invagination and cargo recruitment go hand in hand. Clearly, we can expect these proteins and their partners to act in concert at the site of a nascent vesicle *in vivo*. How this process is achieved remains unclear; neither is it clear to what extent similar activities induce membrane curvature elsewhere in the cell.

Endosome membrane organisation

Once in early endosomes, proteins can be recycled to the cell surface via a rapid or a slow route through recycling endosomes, but a direct route also connects these endosomes with the *trans*-Golgi network (TGN) [12].

Figure 1



Lipid territories in the endocytic pathway. After internalisation from the plasma membrane (PM) via the clathrin pathway, proteins and lipids first appear in early endosomes (EE). Then, these molecules are either recycled to the cell surface, at least in part via recycling endosomes (RE), or are collected within forming ECVs/MVBs. ECVs/MVBs are then translocated on microtubules towards late endosomes (LE), with which they eventually fuse. Molecules that are destined to be degraded are packaged within lysosomes (Lys). The outline shows the distribution of endocytic lipid territories discussed in the text. Whereas PI(4,5)P2 (green) is thought to be predominantly found at the PM and plays a role in the formation of clathrin-coated pits, PI3P (red) and PI(3,5)P2 (black) are believed to be abundant in endosomes. In EEs, PI3P is presumably generated from phosphatidylinositol by a Vps34p complex, and accumulates within ECV/MVB internal membranes. Then, the lipid is either degraded, presumably by myotubularin, or converted into PI(3,5)P2 by PIKfyve — the precise distribution of PI(3,5)P2 is not known. LEs contain large amounts of LBPA (blue), which is generated via an unknown biosynthetic pathway. This lipid is abundant within LE internal membranes, which appear to be distinct from the remaining PI3P-containing vesicles. In addition to LBPA and PI3P domains, LE internal membranes might also contain other membrane domains (yellow), including, perhaps, cholesterol-enriched membranes and rafts.

Ultrastructural studies show that early endosomes are highly pleiomorphic, and contain cisternal, tubular and vesicular regions. While tubules correspond to elements of the recycling endosome, large vesicular regions contain the molecules destined for late endosomes and lysosomes (see outline in Figure 1). These vesicles accumulate membrane invaginations and free vesicles or tubules in their lumen, corresponding to forming endosomal carrier vesicles/multivesicular bodies (ECVs/MVBs). After detaching from early endosomes, ECVs/MVBs are translocated towards late endosomes, with which they eventually fuse. Much like early endosomes, late endosomes also exhibit a complex organisation, containing multivesicular — or multilamellar — regions, but also highly dynamic tubulo-cisternal elements. From late endosomes, some proteins are packaged in lysosomes, while others are recycled back to the TGN. Others routes also connect late endocytic compartments to the cell surface, at least in specialised cells [13,14]. In addition, endosomes and lysosomes are connected to the autophagy pathway [15••]. It is now becoming clear that this mosaic of morphological — and functional — domains reflects differences in the distribution of protein complexes associated to specific lipids and protein–lipid domains within membranes.

Early endosome dynamics

There is no doubt that phosphoinositides play an essential role in membrane traffic by selectively binding well-defined protein domains [16]. Evidence is accumulating that phosphatidylinositol 3-phosphate (PI3P) and its partner proteins (see below) play at least two critical roles in early endosomes — by regulating the dynamics of the compartment and also protein trafficking towards late endocytic stages; PI3P is consistently abundant in early endosomes and ECV/MVB internal membranes compared with late endosomes [17].

The small GTPase Rab5 regulates early endosome membrane dynamics, including docking/fusion and interactions with the cytoskeleton, through interactions with multiple effectors [18]. The active, GTP-bound form of Rab5 is believed to build an effector domain on the membrane, through a self-activation process and the recruitment of both PI3P-binding proteins containing the FYVE motif and PI3-kinases [18] — including the mammalian homologue of the single yeast PI3-kinase Vps34p (see below). In addition, Rab5 and Rab4 are involved sequentially along the recycling pathway and share common effectors, which might coordinate the functions of the corresponding Rab domains in this pathway [19••]. Rab5 effectors also interact selectively with proteins controlling membrane traffic and protein sorting. While the FYVE-domain protein EEA1 binds both syntaxin-6 and syntaxin-13 [18], the Rabaptin-5–Rabex-5 complex interacts with GGAs (Golgi-associated γ-adaptin ear homology, Arf binding proteins) [20•], the Arf-dependent clathrin adaptors involved in selection of TGN cargo. Such crosstalk strongly suggests that Rab5 domains integrate trafficking and sorting functions on early endosomes, like, perhaps, other Rab domains do on different membranes.

Other membrane domains are likely to play a role in early endosome membrane organisation. It has been proposed that early endosomes contain annexin II- and cholesterol-rich regions, and that these are involved in the dynamics of this compartment [12] (see Update). In fact, cholesterol itself seems to be involved in the regulation of protein and lipid trafficking through endosomes [21•]. In addition, endosomes might well contain cholesterol- and glycosphingolipid-rich microdomains or lipid rafts, such as those found in the plasma membrane and implicated in sorting, transport, signalling, and infection [22]. In particular, it has

been proposed that endocytosed glycosylphosphatidylinositol-anchored proteins, which are preferentially raft-associated, undergo sorting in early endosomes, and that their fate depends on their residence time in endosomal rafts [23•]. Interestingly recent studies indicate that clustering of a kinesin in rafts can triggers transport. More specifically, the UNC104 kinesin was found to bind PI(4,5)P2 via its pleckstrin homology (PH) domain, and clustering of Unc104 in PI(4,5)P2- containing rafts promotes transport [24••].

Transport from early to late endosomes

It is now well established that ubiquitination is involved in protein sorting, including into clathrin-coated vesicles [25]. Ubiquitination, together with the ESCRT (endosomal sorting complex required for transport) complexes I to III, also controls the sorting into the MVB of a subset of proteins destined for degradation in yeast [26••]. A similar mechanism is likely to operate in mammalian cells for several, but not all [27], downregulated receptors. For example, Tsg101, the homologue of ESCRT I Vps23p, binds ubiquitin [28•], and its depletion impairs epidermal growth factor receptor (EGFR) trafficking [28•]. Moreover, ubiquitination of EGFR is required for its sorting into the MVB [29]. Interestingly, Tsg101 is also involved in Ebola and HIV retrovirus budding, presumably reflecting the topologically equivalent process of invagination within multivesicular endosomes [26••]. In addition, the FYVE-domain protein Hrs — the homologue of yeast Vps27p that may act upstream of the yeast ESCRT I complex — contains the ubiquitin-interacting motif [30•], and is believed to sort ubiquitinated proteins into clathrin-coated microdomains of early endosomes [31•]. These domains presumably correspond to the newly discovered endosomal bilayered clathrin coat, which is sensitive to PI3-kinase inhibition [32•]. Hrs is also required for degradation of active EGFR and Torso tyrosine kinase receptors in *Drosophila*, and might regulate ECV/MVB biogenesis and the invagination process [33•] (see Update).

Hence, in addition to its role in early endosome dynamics, PI3P also appears to be involved in the ubiquitin-dependent selection of proteins destined for degradation, and perhaps in the biogenesis of the transport intermediates themselves. To what extent PI3P signalling and ESCRT complexes are part of the core transport machinery or control protein sorting is not known (see Update). Neither is it clear whether proteins not destined for degradation or incorporated into ECV/MVB-limiting membrane utilise components of the same machinery.

Sorting nexins

Another phosphoinositide-binding domain, PHOX or PX, was found in $p40^{phox}$ and $p47^{phox}$, and the PX domain of $p40^{phox}$ binds PI3P selectively. This domain is shared by many proteins, including by members of the sorting nexin (SNX) family, and some PX proteins, including some SNX family members, regulate endocytic functions or interact with receptors [34]. Several SNX proteins (Snx1–6 and 13) were found on early or recycling endosomes [34,35], and the PX domain of SNX1 is necessary for this localisation [36,37]. SNX1 — the homologue of Vps5p in yeast, a component of the retromer complex necessary for endosome–TGN recycling — interacts with Hrs and regulates EGFR degradation [12,16]. Interestingly, mice lacking SNX1 or SNX2 are viable, but embryos lacking both proteins are not, indicating that the proteins are redundant and that their function is vital [38]. The phenotype of $Snx1^{-/-}/Snx2^{-/-}$ embryos resembles that of embryos lacking another retromer homologue, Hbeta58/Vps26p. These show no obvious endocytic defect, but apical compartments in the yolk-sac visceral endoderm are altered, leading authors to conclude that SNX1/SNX2 act in the mammalian retromer pathway. SNX9 interacts with the AP2 ear domain [39], and with ACK2, the activated Cdc42-associated kinase 2 — an interaction that might also regulate EGFR degradation [40]. SNX9 also interacts with *Drosophila* ACKs (a Cdc42-associated tyrosine kinase) and the actin regulator Wiskott–Aldrich syndrome protein (WASP) protein in the regulation of axonal guidance [41]. Future work is required to understand the precise functions of PX proteins — and their possible relationships to FYVE-domain proteins — in the regulation of signal transduction, protein sorting and membrane transport.

Phosphoinositide kinases and phosphatases

PI3P is presumably generated on early endosomes, at least in part, by Vps34p, since this kinase is a Rab5 effector [18]. Vps34p, however, was found in two protein complexes involved in autophagy and endosome-to-Golgi retrograde transport [42,43]. Since Vps34p functions in yeast Golgi-to-vacuole transport, additional complexes may exist, and each might generate a specific pool of PI3P within specific microdomains [43]. In addition, the yeast FYVE protein Fab1p — a PI3P 5-kinase that generates PI(3,5)P2 — is essential for vacuole morphology and may regulate sorting into the vacuole lumen [44]. The mammalian Fab1p homologue PIKfyve, which binds PI3P, was found on late endosomes in a PI3P- and FYVE-dependent manner [45], and its capacity to generate PI(3,5)P2 is involved in the maintenance of endosome morphology [46]. The precise roles of PIKfyve and PI(3,5)P2, as well as the relationships with PI3P and PI3P-binding proteins, remain to be determined. Progress is also being made in studying the role of 3-phosphatases, including in human diseases [47]. Myotubularin (MTM1), which is mutated in X-linked myotubular myopathy, dephosphorylates an endosomal pool of PI3P, in contrast to myotubularin-related MTMR2, which is mutated in the type 4B Charcot-Marie-Tooth disease [48]. MTMR3, which contains a FYVE domain, like MTMR4, but does not seem to localise to endosomes, exhibits 3-phosphatase

activity towards both PI3P and PI(3,5)P2 and might play a role in autophagy [49].

Late endosomes: lipid domains and proteins

Late endosomal membranes undergo a major remodelling process in animal cells (Figure 1). The internal membranes of their multivesicular elements accumulate large amounts of the poorly degradable phospholipid lysobisphosphatidic acid/bis(monoacylglycerol) phosphate (LBPA) [12,50]. LBPA turnover might depend on a recently described phospholipase A2 [51]. This lipid could facilitate the membrane invagination process, since it has an inverted cone shape and may be synthesised *in situ*. Internal membranes of multivesicular compartments also contain cholesterol [52•], and cholesterol-enriched detergent-resistant membranes (rafts) were isolated from late endosomes [23•]. Cholesterol- and LBPA-containing internal membranes might correspond to different domains, since late endosomes seem to contain more than one type of internal membranes [17,53]. To what extent the biogenesis and dynamics of late endosome inner membranes are regulated by the same mechanisms as those operating during ECV/MVB formation is not known.

In addition to lipids, proteins also show a restricted distribution within late endosomes. The glycoproteins Lamp1 and Lamp2, and MLN64, a homologue of the mitochondrial steroidogenic acute regulatory protein (StAR), are only found on the membrane that encloses this multivesicular compartment, referred to as the limiting membrane, and not within the lumenal vesicles and invaginations [12]. By contrast, proteins destined to be degraded often accumulate within ECV/MVB internal membranes — and presumably remain lumenal until degradation is completed. Since LBPA and other negative phospholipids could facilitate glycolipid degradation [54], and since LBPA itself is poorly degradable, one function of LBPA-membranes might be to present lipids and proteins to the hydrolytic machinery. But internal membranes of late endosomes also contain proteins that are not destined to be degraded [12], including tetraspanins [55]. These are also found on the cell surface, where they may form microdomains enriched in MHC class II in antigen-presenting cells [56]. In these cells, MHC class II molecules are also abundant within internal membranes of late endosomes and lysosomes. From this intralumenal location, MHC class II are transported to the cell surface via tubules [57] that may form at the expense of internal membranes [55]. How this process relates to the release of internal membranes into the medium (exosomes) after direct fusion of multivesicular endosomes with the plasma membrane is not known [55].

Late endosomes: trafficking and dynamics

Protein and cholesterol transport through late endosome internal membranes is inhibited when interfering with LBPA-membranes using endocytosed anti-LBPA antibodies — thereby mimicking the cholesterol-storage disorder Niemann–Pick type C (NPC) [12,50,58]. Conversely, cholesterol accumulation in late endosomes inhibits protein trafficking, including in NPC cells. Moreover, late endosome motility is impaired after inhibition of LBPA functions or cholesterol accumulation, by interfering with the membrane–cytosol cycle of the small GTPase Rab7 [59••]. Similarly, motility is inhibited in NPC cells [59••,60,61].

These observations underscore the existence of a mechanism by which the membrane lipid composition might control organelle dynamics, via the membrane–cytosol cycle of a key regulator.

Late endosomes form dynamic networks [59••] that contain distinct morphologically visible regions [12] and membrane domains selectively enriched in Rab7 or Rab9 [62•] — two GTPases that regulate late endocytic traffic and late endosome-to-TGN recycling, respectively. This, together with the findings that different types of membranes can be isolated from late endosomes [23•,53], support the notion that late endosomes are formed from a mosaic of regions and membrane domains that differ both structurally and functionally [1]. In line with this view, different subcompartments were proposed to be involved in lipid loading onto CD1D, which is one of the isoforms of CD1 proteins that bind and present lipid antigens to T cells [63]. LBPA membranes might have turnpike functions in this network, as they seem to be involved both in transport and degradation. Transport defects in NPC and perhaps other lipid-storage disorders could result from a collapse of this mosaic architecture, leading to mixing of domains and eventually to transport inhibition.

Cholesterol transport and storage

How cholesterol is exported from late endosomes is not yet clear. The cholesterol-storage disorder NPC is caused by mutations in NPC1, a permease that facilitates *trans*-bilayer fatty acid transport, or less frequently in NPC2, a secreted cholesterol-binding protein [64]. Export might also depend on MLN64 [65]. In addition, overexpression of Rab7 or Rab9 partially corrects the NPC phenotype [66]. It is not clear how these GTPases act; it might be via different pathways, since they have different functions and localise to different domains. Moreover, Rab7 overexpression inhibits motility by causing the paralysis of late endosomes in the pericentriolar region [59••], presumably via the Rab7 effector Rab-interacting lysosomal protein (RILP) [67,68]. Interestingly, the Rab9 effector TIP47 is homologous to the adipophilins of the lipid droplets [69], a storage site for cholesterol esters and triglycerides. Whether TIP47 is present in lipid droplets is controversial, however. But other studies have also uncovered an unexpected relationship between lipid

droplets and late endosomes. Although caveolins are normally found in caveolae at the plasma membrane, caveolin-2β and a truncated caveolin-3 mutant are present in lipid droplets [70]. Concomitantly, this mutant causes NPC-like cholesterol accumulation in late endosomes [71]. It is therefore possible that intracellular transport of free cholesterol and storage of cholesterol esters are connected functionally, via an as yet undiscovered mechanism.

Conclusions

Different phosphoinositide territories distribute within membranes at sequential stages of the endocytic pathway, from the plasma membrane to late endosomes (Figure 1). A downregulated receptor seems to pass — like a baton in relay racing — from one territory to the next through direct or indirect interactions with proteins or proteins complexes that bind each phosphoinositide specifically. In addition, endosomes also contain not only morphologically visible, and functionally defined, regions, but also different membrane domains, each with a characteristic lipid composition. Amongst these, LBPA-membranes, and perhaps other membrane domains, play a role in traffic, presumably because they preferentially incorporate some proteins and lipids, or interact selectively with soluble factors on the lumenal or cytoplasmic face of the bilayer. Hence, protein sorting and membrane transport seem to be controlled, at least in part, by the mosaic organisation of endosomal membranes — an organisation likely to also play a role in other cell-related processes, including development and infection [58,72,73]. A future challenge will be to determine, in addition to the precise functions of individual players, what mechanisms orchestrate the crosstalk between membranes domains and lipid–protein complexes at each stage of the pathway.

Update

In line with the notion that annexin II and cholesterol-rich regions are involved in early endosome membrane dynamics, recent work suggests that annexin II interacts physically with cholesterol, and that such annexin-II–cholesterol platforms regulate the onset of the degradation pathway, by controlling ECV/MVB biogenesis in animal cells [74].

Downregulation of Hrs with small interfering RNA has shown that Hrs also plays a role in the membrane invagination process that accompanies ECV/MVB formation [75], consistent with previous findings [33•]. While both Hrs and PI3P signalling control downregulated receptor sorting, they might not be necessary for bulk transport from early to late endosomes (A Petiot, J Fauré, H Stenmark and J Gruenberg, unpublished data), perhaps suggesting that the membrane invagination process can be uncoupled from vesicle formation.

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