

# ALIX-ing phospholipids with endosome biogenesis

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## Summary

Endosomes, which comprise a diverse population of membrane vesicles and tubules, sort proteins and lipids to various cellular destinations. The organization and functions of these pleiomorphic cellular organelles have been extensively studied. Matsuo et al.<sup>(1)</sup> now provide new exciting evidence on the role of lysobisphosphatidic acid (LBPA), a resident phospholipid of internal vesicles of the late endosome, in the control of membrane invagination and endosome biogenesis. In vivo, LBPA functions are controlled by the adaptor protein Alix and depend on pH gradients along the endosomal compartments. *BioEssays* 26:604–607, 2004.

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## Introduction

The endosome is a complex cellular apparatus composed of numerous membrane vesicles, tubules and lamellas that decide the fate of proteins and lipids from biosynthetic and endocytic pathways.<sup>(2)</sup> In the endocytic pathways, internalized molecules are initially delivered into early endosomes, which segregate receptors destined for recycling into long tubular extensions and return them to the plasma membrane.<sup>(2,3)</sup> The remaining central vacuole, which is enriched in molecules targeted for a degradation pathway, moves towards the centre of the cell to become the late endosome. This is accomplished by either a fusion or a maturation process that creates a pleomorphic set of late endosomal organelles containing external limiting membranes and a complex system of poorly characterized internal membrane invaginations in their lumen.<sup>(3)</sup> These structures, also known as multivesicular bodies (MVBs), function as an important relay station between secretory and degradation pathways by delivering trafficking cargoes for degradation in the lysosome or by redirecting recycling proteins.<sup>(2)</sup> Whether these interior membrane structures contribute to different MVB functions is not yet fully understood. It is known, however, that both limiting and internal membranes undergo dynamic remodelling by numerous cycles of fusion and fission events.<sup>(3)</sup> It was originally

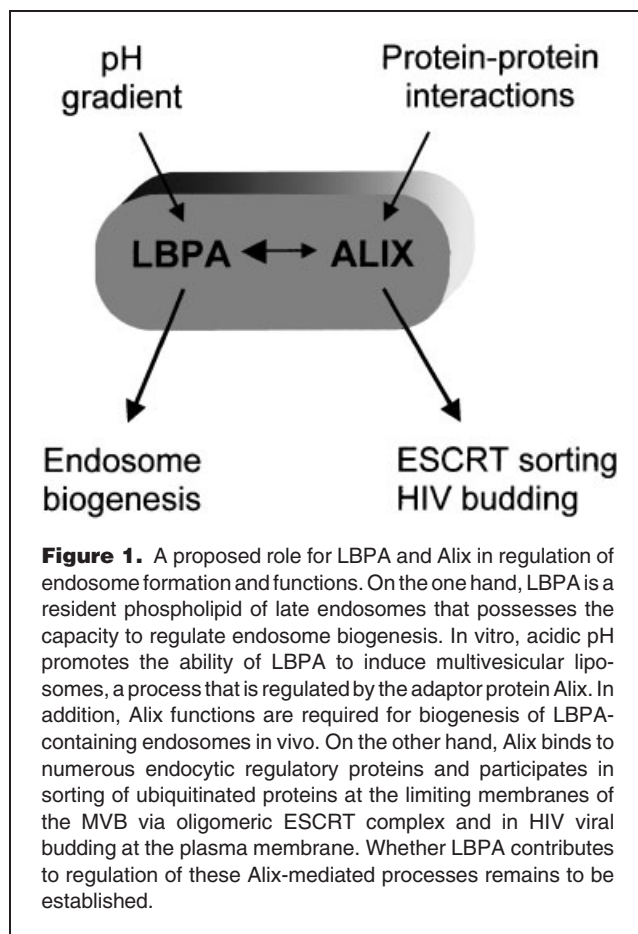
thought that proteins en route to degradation are sorted at the limiting membranes of the MVBs and that only proteins targeted for degradation will be found in internal membranes. Recent findings showing that the recycling receptors are also present on internal membranes, whereas lysosomal marker proteins are found on the limiting membranes, point to the existence of an interconnected network of membranes that transport proteins to the lysosome as well as out of the endosome.<sup>(3)</sup>

It has been known for a long time that lipids play important roles in endosome biogenesis, not only because they are the major structural constituents of cell membranes, but also because they actively participate in the organization and formation of the vacuolar apparatus.<sup>(4)</sup> Cellular membranes contain numerous phospholipids and some of them are specifically distributed within distinct parts of endosomal membranes, where they form microdomains empowered with distinct functions.<sup>(4)</sup> For example, the raft lipids cholesterol and sphingomyelin participate in protein sorting along the recycling route.<sup>(5)</sup> Phosphoinositol-4,5-bisphosphate PI(4,5)P<sub>2</sub> is enriched in the plasma membrane where it recruits and activates adaptor protein 2 (AP-2),<sup>(6)</sup> while phosphoinositol-3-phosphate (PI3P) is predominantly found at early stages of the endocytic pathway and interacts specifically with numerous proteins that regulate endocytic transport.<sup>(7)</sup> In addition, two populations of membranes containing either phosphatidylcholine (PC) or lysobisphosphatidic acid (LBPA) as their major phospholipids can be distinguished in the interior vesicles of the MVB.<sup>(8)</sup> A speculative model has been put forward whereby membranes rich in PC, which has a preference for fluid regions, may participate in curvatures of internal vesicles, whereas LBPA, a small cone-shaped lipid with fusogenic properties, may be involved in the regulation of internal membrane organization and their functions.<sup>(8)</sup> In a recent paper, Matsuo et al provide further molecular details on how LBPA may regulate formation of internal vesicles and control late endosome biogenesis in vivo (Fig. 1).<sup>(1)</sup>

## LBPA—a marker or a ruler of the endosome biogenesis?

LBPA is a structural isomer of phosphatidylglycerol that is highly enriched in internal membranes of the MVB.<sup>(8,9)</sup> It was initially detected by an antibody that specifically labelled the late endosomal compartment.<sup>(10)</sup> Subsequent lines of

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evidence have shown that LBPA is not only an endosomal marker, but also an important player that regulates the dynamics and functions of the internal membranes inside the MVBs. Actually, antibodies against LBPA interfered with trafficking through the late endosome<sup>(10,11)</sup> and caused cholesterol accumulation, mimicking the cholesterol-storage disorder Niemann-Pick type C.<sup>(11)</sup> Moreover, LBPA exhibited unique pH-dependent ability to fuse membranes in liposomes<sup>(8)</sup> and was shown to be critical for the correct sorting of transmembrane proteins, such as the mannose-6-phosphate receptor from the late endosomes to the trans Golgi network.<sup>(10)</sup>

In their report, Matsuo et al. move a step further and demonstrate that LBPA has an intrinsic ability to induce formation of vesicles within acidic liposomes.<sup>(1)</sup> Liposomes were prepared in vitro to contain a phospholipid composition similar to that of late endosomes and were incubated at pH 5.5 during the liposome assembly process. The external pH was subsequently neutralized, thus creating a pH gradient similar to the late endosome in vivo. Under these conditions, 2,2'-dioleoyl LBPA (the most abundant form in the internal membranes) but not 3,3'-LBPA (LBPA  $\beta$ -isoform) or semi-LBPA (found in Vaccinia virus and perhaps in Golgi), could

induce multivesicular liposomes.<sup>(1)</sup> Importantly, 2,2'-LBPA failed to promote vesicular formation in the absence of a pH gradient or in the presence of anti-LBPA antibodies. The LBPA-induced bilayer invagination process was also reconstituted in liposomes that were prepared at neutral pH and then acidified with a protonophore.<sup>(1)</sup>

The idea that LBPA regulates membrane invagination is appealing, but there are many uncertainties on how LBPA can control these processes. It is thought that in pure lipid preparations membrane curvature is driven by lipid asymmetry across the lipid bilayer.<sup>(12)</sup> This would imply that LBPA exerts its effects via localization to specific sites in liposome membranes and that it is not diffused out in the surrounding membranes. Differential LBPA distribution might be achieved by retention of phospholipids during repeated cycles of vesicle or tubule formations.<sup>(12)</sup> Moreover, LBPA is a negatively charged molecule that is predicted to have a cone-shaped structure with a small head group.<sup>(9)</sup> Cone-shaped or type II lipids are believed to form hexagonally arranged cylinders in vitro with the polar head groups lining a central aqueous channel.<sup>(12)</sup> From such geometric considerations, it is clear that cone-shaped lipids will prefer to reside in membranes with changes in curvatures, rather than in planar bilayers. Consistent with this, LBPA generally forms non-bilayer structures when present in high local concentrations.<sup>(1,8)</sup> However, it is important to emphasize that the curvature preference of LBPA can largely be influenced by pH, temperature and salt concentration. Actually, liposomes containing LBPA stimulated more internal vesicles formation at pH 5.5 and at 37°C than at neutral pH and 4°C.<sup>(1,8)</sup> The authors have proposed that the pH-induced assembly of LBPA molecules may locally deform the bilayer, thus leading to membrane invagination. The conclusion that LBPA can drive the formation of multivesicular liposomes in vitro naturally raises the question of whether the same basic principles also operate in vivo. Initial evidence indicates that the situation in living cells is much more complicated. At present little is known about the molecular mechanisms underlying the assembly of LBPA microdomains in membrane bilayers and whether LBPA partitioning can actively control membrane changes in vivo. Some preliminary evidence indicates that LBPA may be asymmetrically localized in endosomal membranes in cells. The inclusion and maintenance of LBPA in internal membranes of MVBs might be achieved during its uneven synthesis from a lipid precursor or by an active transport of lipids across the membrane bilayer.<sup>(13)</sup> It is known that phospholipids with polar head groups, like LBPA, cannot cross a pure lipid bilayer, whereas neutral lipids can rapidly flip across membrane bilayers.<sup>(12)</sup> Therefore, the flip-flop movement of LBPA, if it exists in cells at all, is likely to involve phospholipid transfer-exchange proteins such as translocases.<sup>(13)</sup> Importantly, such an active transport is expected to be under the control of local signals that can actively remodel membranes depending on the functional

status of the cell. An alternative theory, which is offered by the authors, proposes that the transport of LBPA molecules may be caused by the protonation of LBPA phosphate groups. This hypothesis is consistent with results demonstrating that protonation of phosphatidylcholine can promote its flipping from one leaflet to the other.<sup>(14)</sup> In general, there is a sufficient body of evidence to conclude that LBPA has a potency to modulate the structural and functional properties of lipid bilayers.

### The role of Alix in regulation of LBPA functions

In living cells, it is well established that the formation of endosomal vesicles requires interactions between the membrane lipids and the cytosolic proteins that coat and deform the membrane.<sup>(2,15)</sup> Sorting of internalized membrane proteins to endosomes and lysosome is partly mediated by linear peptide signals within the cytosolic domains of transmembrane proteins.<sup>(15)</sup> In addition to peptide motifs, the attachment of ubiquitin (Ub) to receptors serves as an essential signal required for recruitment of a given protein cargo and its sorting into inward budding vesicles from either the plasma membrane or the MVB.<sup>(16,17)</sup> Molecular details of membrane invagination and fission at the plasma membrane are relatively well established and include an elaborate network of proteins able to bind and curve membranes.<sup>(15)</sup> More recently, studies in yeast and mammalian cells have shown that oligomeric protein complexes containing the adaptor proteins Hrs and ESCRT complexes mediate recognition and sorting of cargo at the limiting membranes of the MVBs to inward budding vesicles.<sup>(18,19)</sup> This process is topologically inverted as compared to receptor internalization at the plasma membrane. In this context, the orientation of cytoplasmic part of receptors is facing the centre of the budding vesicle and it is unclear whether cytoplasmic protein complexes contribute at all to the inward budding and fission of internal vesicles. Matsuo et al. put forward an interesting hypothesis suggesting that LBPA may be involved in these processes via its interaction with cytosolic protein complexes.<sup>(1)</sup> By incubating liposomes with the cellular cytosol, they isolated five proteins that specifically associated with LBPA-containing liposomes. Using tandem mass spectrometry, one of them was identified as Alix, a protein known to interact with many proteins involved in membrane invagination in the MVBs.<sup>(1)</sup>

Alix (also known as AIP1) was originally discovered as a binding partner of the Ca<sup>2+</sup>-binding protein ALG-2 (apoptosis-linked gene 2) involved in apoptosis pathways in neuronal cells.<sup>(20)</sup> Alix also associates with other cellular proteins involved in signal transduction such as CIN85/SETA,<sup>(20)</sup> which regulates endocytosis of ubiquitinated tyrosine kinase receptors<sup>(21–23)</sup> and endophilins.<sup>(24)</sup> The latter are lysophosphatidic acid acyltransferase and BAR domain-containing proteins capable of modifying the lipid bilayer curvature during

endocytosis.<sup>(25,26)</sup> Furthermore, the yeast AIP1 homolog Bro1 functions in concert with components of the ESCRT machinery to regulate MVB formation.<sup>(27)</sup> This evidence suggests, although does not yet prove, that Alix may be directly involved in the regulation of membrane dynamics in cells.

Consistent with the aforementioned hypothesis, Matsuo et al show that the addition of a recombinant Alix to LBPA-containing liposomes in the absence of cytosol leads to inhibition of MVB formation whereas its depletion by specific antibodies stimulates MVB formation.<sup>(1)</sup> In contrast, down-regulation of Alix by siRNA leads to a lower number of acidic late endosomal compartments and reduces the level of LBPA.<sup>(1)</sup> Moreover, the Vesicular Stomatitis Virus (VSV) infection was inhibited following silencing of Alix.<sup>(1)</sup> This may be caused by a decrease in acidic late endosome, which is required to trigger fusion of the VSV envelope with endosomal membranes. An alternative scenario, not mutually exclusive with the former, is that Alix is also required for the back fusion of internal membranes with limiting membranes in the MVB. Based on these data, the authors conclude that Alix may be involved in both fission and fusion of late endosomal membranes.

One simple explanation of these findings is that Alix can directly associate with LBPA lipids and modulate their capacity to change membrane curvature and affect fusogenic properties of membranes. This also suggests that, in living cells, the inward budding and fission processes are controlled by transient and possibly direct interactions between LBPA membranes and Alix. If this hypothesis holds true, LBPA and Alix are expected to function on the cytoplasmic side of the membrane. However, it is very unclear how Alix–LBPA can mechanistically promote membrane invagination and fission under these conditions. Since Alix participates in the control of sorting signals at the limiting membranes of the MVBs, the disruption of Alix functions may very well affect this process rather than the dynamics of the internal membranes.

There are many future challenges in this field. A clear determination of the biochemical and biophysical properties of LBPA–Alix interactions is needed. An interesting question is also whether LBPA may affect the ability of Alix to regulate critical steps of HIV budding from either the plasma membrane or intracellular membranes. In fact, Alix was shown to bind both the HIV Gag protein, as well the TSG101 and CHMP4, components of the ESCRT machinery, in order to promote pinching off or fission of membrane and the release of viruses from the cell surface.<sup>(28–30)</sup> This process is mechanistically comparable to inward budding of internal vesicles of the MVB and it is therefore possible that the inhibition of Alix interactions with ESCRT complexes in turn blocks the formation of the internal vesicles in late endosomes. It is important to note that Alix is highly enriched in exosomes, which are specialized secreted membrane vesicles found in immune cells.<sup>(31)</sup> These are originally formed by inward budding from the limiting

membrane into the lumen of the MVB and are secreted upon fusion of limiting membranes of multivesicular endosomes with the plasma membrane.<sup>(31)</sup> It remains to be established if there is any contribution of LBPA in biogenesis of exosomes. Finally, it will be essential to precisely analyze the intracellular compartments with which Alix associates and their relation to LBPA membrane subdomains to get a better insight into many of these questions.

### Conclusions

The work of Matsuo and colleagues is an exciting first step in discovering details of protein–lipid interactions that control endosome maturation and organization. Two important messages emerge from this paper. The first is that LBPA can actively drive the formation of membrane invaginations and the second is that Alix-containing protein complexes play critical roles in modulating the former activity *in vivo*.<sup>(1)</sup> Better understanding of the Alix–LBPA interplay will very likely provide us with a deeper insight into regulation of membrane dynamics in living cells.

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