Separation and Characterization of Late Endosomal Membrane Domains*

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Very little is known about the biophysical properties and the lipid or protein composition of membrane domains presumably present in endocytic and biosynthetic organelles. Here we analyzed the membrane composition of late endosomes by suborganellar fractionation in the absence of detergent. We found that the internal membranes of this multivesicular organelle can be separated from the limiting membrane and that each membrane population exhibited a defined composition. Our data also indicated that internal membranes may consist of at least two populations, containing primarily phosphatidylcholine or lysobisphosphatidic acid as major phospholipid, arguing for the existence of significant microheterogeneity within late endosomal membranes. We also found that lysobisphosphatidic acid exhibited unique pH-dependent fusogenic properties, and we speculated that this lipid is an ideal candidate to regulate the dynamic properties of this internal membrane mosaic.

Evidence is accumulating that some lipids contribute to the organization and functions of the vacuolar apparatus. Microheterogeneity of the plasma membrane could be studied using morphological and biophysical approaches, because the cell surface is readily accessible to probes and agents added from the medium. These studies revealed that, in addition to the fluid glycerophospholipid-rich regions of the bilayer, the plasma membrane also contains liquid-ordered regions rich in cholesterol and glycosphingolipids (rafts), in which glyco-sphingosylphosphatidylinositol-anchored proteins and double-acylated proteins seem to preferentially partition. Lipid rafts are believed to act as cell surface platforms involved in internalization, signaling, and infection by pathogens and also to facilitate protein sorting in the biosynthetic pathway (1–3). However, it has been extremely difficult to study putative intracellular lipid domains without using perturbing agents, e.g. methyl-β-cyclodextrin to deplete cholesterol, or detergents, including CHAPS1 (4, 5), or Triton X-100 (6). Hence, very little is known about the biophysical properties and lipid or protein composition of membrane domains presumably present in endocytic and biosynthetic organelles.

In the endocytic pathway, molecules internalized into early endosomes are either recycled back to the plasma membranes or transported to late endosomes and lysosomes for degradation. Evidence is accumulating that some lipids are not randomly distributed in endosomal membranes along these recycling and degradation routes, contributing to the notion that endosomes contain a mosaic of structural and functional membrane domains (7). In particular, raft lipids seem to be abundant in recycling endosomes, at least in some cell types (8). However, lipid analogs with a preference for ordered membrane domains were reported to be transported toward late endosomes, in contrast to those with a preference for more fluid domains (9). Phosphatidylinositol 3-phosphate, which interacts with proteins containing a FYVE or a PX domain (10, 11), appears to be more abundant at early stages of the endocytic pathway (12), and lysobisphosphatidic acid (LBPA) is restricted to late endosomes (13). Immunogold labeling of cryosections shows that LBPA is abundant within the internal membranes of this multivesicular or multilamel-lar organelle (13) and cannot be detected on the cytoplasmic surface of the limiting membrane (14). In this paper, we report that late endosomal membranes can be separated without detergents into distinct populations, each with a unique composition, corresponding to limiting and internal membranes. Our data also suggest that internal membranes can be subfractionated into more than one population, including LBPA-rich membranes, and that LBPA itself exhibits unique pH-dependent fusogenic properties. We conclude that late endosomal internal membranes contain different mem-

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1 The abbreviations used are: CHAPS, 3-[(3-cholamidopropyl)dimethy-

ylammonio]-1-propanesulfonic acid; LBPA, lysobisphosphatidic acid;

BHK, baby hamster kidney; β-BODIPY FLCL2-HPC, 2,4,4-difluoro-

5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoyl-1-hexade-

noyl-sn-glycerol-3-phosphocholine; DOPE, dioleoylphosphatidylcholine;

PL, phospholipid; PS, phosphatidylserine; PI, phosphatidylinositol; PE,

phosphatidylethanolamine; FC, phosphatidylcholine; VSV, vesicular

stomatitis virus; GFP, green fluorescent protein.
brane domains and that their dynamic interplay is regulated by LBPA.

MATERIALS AND METHODS

Cells and Reagents—2-(4,4'-Diffuoro-5,7-dimethyl-4-bora-3a,4a-
diaza-s-indacene-3-dodecanoyl)-1-hexadecanoyl-en-glycerol-3-phos-
pholcholine (β-BODIPY FLC12-HPC) was from Molecular Probes (Eugene, OR). Dioleoylphosphatidylcholine (DOPC) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). DEAE-Sephadex A-25 was from Amersham Biosciences AB. Pre-swollen carboxymethyl cellulose CM52 was from Whatman. Monolayers of baby hamster kidney (BHK) cells were grown and maintained as described. When necessary to determine the protein composition of late subfractions, cells were metabolically labeled by incubation for 16 h before the experiment with 0.5 mCi/ml [35S]methionine/[35S]cysteine ExpreSS label (PerkinElmer Life Sciences) in low Met/Cys medium (1.5 mg/liter Met, 3.1 mg/liter Cys). To determine the phospholipid (PL) composition of endosomal subfractions, cells were metabolically labeled for 16 h with 32P, or with 14Cacetate. The monoclonal antibodies against LBPA (6EC4) and Lamp 1(A1A) have been described.

Purification of 3,3′- and 2,2′-LBPA—3,3′-LBPA was purified from BHK lipids extracts by preparative TLC after a silica gel (Intersil, Sigma, and DEAE-Sephadex A-25 column chromatography); the purified lipid migrated as a single spot on high performance TLC plates and was resistant to phospholipase A2 digestion. 2,2′-LBPA was purified using DEAE Sephadex A-25 and CM52 column chromatography.

Gas-Liquid Chromatography and Mass Spectrometry—Phospholipid Analysis—Cells were labeled to equilibrium for 16 h with [32P] and then fatty acids were converted to their methyl esters and analyzed by gas-liquid chromatography. The table shows the major fatty acid species as a percentage of the total amounts of fatty acids in LBPA.

| Fatty acid     | %     |
|---------------|-------|
| Palmitic acid | 2.8   |
| Palmitoleic acid | 3.4  |
| Stearic acid  | 1.9   |
| Oleic acid    | 91.8  |

The first direction was run with chloroform, methanol, 32% ammonia (65:35:5, v/v) and the second direction with chloroform/aceton/methanol/acetic acid/water (50:20:10:12:5.5, v/v). Radioactive lipids were detected by autoradiography and then quantified using the Molecular Imager System (Bio-Rad GS-363).

RESULTS

2,2′-Dioleoyl-tysocephosphatidic Acid—Biochemical analysis of LBPA purified from BHK cells revealed that oleic acid accounted for >90% of LBPA fatty acyl chains (Table I). This was confirmed by mass spectrometry, because the most abundant ion peak was at m/z 773.54 (theoretical value, 773.53), corresponding to [M − H]− of LBPA containing two oleic acids (Fig. 1). These data show that >90% of the lipid is present as dioleoyl-LBPA in BHK cells. However, the position of the fatty acyl chains is unclear, either the β- (24) or the α-position (25, 26) of the glycerol backbone. Because the β-position is thermodynamically unstable, fatty acids may migrate to the α-position during purification by silica column chromatography (27). Indeed, LBPA purified by silica chromatography (15) was completely degraded by R. arrhizus lipase (Fig. 2A), which is well known to cut preferentially at the α-position. In contrast, an analysis of total lipids extracted from late endosomal fractions, however, showed that LBPA was fully resistant to lipase, whereas PC and PE were almost completely degraded (Fig. 2, B and C). These data strongly suggest that acyl chains are predominantly esterified to the β-position in vivo and that LBPA is thus mostly present in living cells as the 2,2′-dioleoyl isoforin (herein referred to as 2,2′-LBPA, Fig. 2D).
LBPA is a structural isomer of phosphatidylglycerol with an uncommon stereoconfiguration, which presumably accounts for its resistance to phospholipase and degradation (28). The lipid is related to cardiolipin and is predicted to be cone-shaped. In addition, the presence of free hydroxyl groups at the 3,3-position (Fig. 2D) may influence its biophysical properties. Indeed, these groups are presumably not buried in the hydrophobic regions of the bilayer but exposed on its outer surface, and thus likely to face the acidic endosomal milieu. We therefore measured the pH-dependent fusion properties of donor liposomes containing 74 mol % dioleoylphosphatidylcholine (DOPC), 21 mol % 2,2'-LBPA, and 5 mol % BODIPY FLC12-HPC, as a fusion tracer, with acceptor liposomes containing 67 mol % DOPC and 33 mol % 2,2'-LBPA. As shown in Fig. 3, these liposomes were highly fusogenic at pH 5.6, a value in the range of late endosomal and lysosomal pH (29). Fusogenicity decreased at pH 6.0 and was essentially abolished at pH 6.5 or 7.3. In contrast, liposomes lacking LBPA did not show pH-dependent fusion activity (not shown). These observations show that 2,2'-LBPA is endowed with unique properties and suggest that the lipid might contribute to the dynamic properties of late endosomal internal membranes.

Subfractionation of Late Endosomal Lipids—Next, we investigated whether late endosomal lipids could be fractionated into different membrane populations, using a "suborganellar fractionation" protocol. We have shown previously (13, 15, 16) that late endosomes are easily separated from other endosomal or biosynthetic membranes by floatation in a step sucrose gradient. These purified late endosomal fractions do not contain the mitochondrial lipid cardiolipin and are depleted in phosphatidylserine (PS), cholesterol, and sphingomyelin, which are enriched in recycling endosomes (8, 13). Purified late endosomes contain a defined subset of phospholipids (PLs) and are highly enriched in LBPA. We showed previously (13, 30) that LBPA accounts for ~0.5% of cellular 32P-labeled PLs and ~14% of late endosomal 32P-PLs (see Table II). In order to ensure that these values were not biased by the lipid phosphate group turnover, experiments were repeated after metabolic labeling with [14C]acetate. As shown in Table II, very similar values were obtained when comparing 32P- and 14C-labeled lipids.

Purified late endosomes were then ruptured under hypotonic conditions by 3 freeze/thaw cycles. Endosome rupture was assessed by measuring the amounts of endocytosed horseradish peroxidase, a content marker, that remained endosome-associated after sedimentation at high speed (latency) (31). After the treatment, latency was reduced to 10% of the untreated control (not shown). This treatment was gentle, because 3 freeze/thaw cycles had hardly any effect on the latency of an early endosomal content marker (not shown). Ruptured late endosomes were then loaded at the bottom of a continuous sucrose gradient (8–40%) and centrifuged to equilibrium for 16 h at 4 °C. After metabolic labeling with 32P, 32P-PLs distributed across the entire gradient (Fig. 4) with three peaks in fractions 4 (d = 1.0655 g/cm³), 7–8 (d = 1.1031 g/cm³), and 12 (d = 1.1654 g/cm³). Intact late endosomal lipids distributed on the gradient as a single peak (d ~1.0772 g/cm³), at the expected density of 32159.

FIG. 1. Mass spectrum of LBPA. LBPA was purified from BHK cells and then analyzed by mass spectrometry. The ion peak at m/z 773.54 corresponds to [M – H]⁺ of LBPA containing two oleic acids.

FIG. 2. Structural analysis of LBPA. A, LBPA purified on silica columns was treated with lipase and then analyzed by thin layer chromatography. B, total lipids from late endosomal fractions were prepared (without column chromatography) and analyzed by two-dimensional TLC. C, total late endosomal lipids were analyzed as in B, after lipase treatment as in A. Whereas PC and PE were almost completely degraded after lipase treatment, LBPA was fully resistant. The arrow points at a lipid appearing after lipase treatment, presumably lys-PC. D, structure of 2,2'-dioleoyl-LBPA.
late endosome (not shown). As a control, early endosome \(^{32}\text{P}\) -labeled PLs after the same freeze/thaw cycles distributed as a single peak in fraction 7 (\(d = 1.1031 \text{ g/cm}^3\)), at the expected density of early endosomes (see Fig. 6). These experiments show that late endosomal lipids can be subfractionated into different membrane populations.

**Lipid Composition of Suborganellar Fractions**—We then analyzed the PL composition of each fraction, and we found that the distribution of each PL varied significantly across the gradient (Fig. 5A). Phosphatidylinositol (PC) was mostly\(^{32}\text{P}\) found in fractions 3–5 at \(d = 1.0571–1.0772 \text{ g/cm}^3\). In these fractions, PC accounted for >70% of the total PLs, while accounting for \(\approx 48\%\) of total \(^{32}\text{P}\)-containing PLs in late endosomes (Table II) and other membranes (13). Phosphatidylethanolamine (PE, 30%) and phosphatidylinositol (PI, 40%) were recovered predominantly in fraction 7 at \(d = 1.1031 \text{ g/cm}^3\). In this fraction, PE and PI accounted for 42 and 16% of total PLs, while representing roughly 19 and 4% of total late endosomal \(^{32}\text{P}\)-PLs, respectively (Table II), again much like in other membranes (13). Finally, the bulk of LBPA (~40%) was found in fractions 11 and 12 (\(d = 1.1545–1.1654 \text{ g/cm}^3\)), where it was highly enriched, corresponding to \(\approx 60\%\) of total PLs in these fractions, and representing \(\approx 15\%\) of total late endosomal PLs (Table II). Some LBPA was also found in fraction 1, perhaps reflecting LBPA partial association with neutral lipids, in particular cholesterol esters (30). This PL distribution did not result from the fragmentation protocol, because it was essentially identical after osmotic rupture of late endosomes (not shown). When using early endosomal fractions as starting materials, no difference in the PL composition of fractions across the peak (fraction 7, \(d = 1.1031 \text{ g/cm}^3\)) was detected (Fig. 6).

This analysis thus indicates that different membrane populations could be separated after late endosome fragmentation (Figs. 4 and 5B). Fractions 3 and 4 (\(d = 1.0571–1.0655 \text{ g/cm}^3\)) contained mostly PC (>70%), some PE and PS (7–10%), and low (<5%) levels of other PLs. The peak in fraction 7 (\(d = 1.1031 \text{ g/cm}^3\)) exhibited a complex composition and contained all PLs, but mostly PE (42%), PC (23%), and PI (16%). For comparison, Fig. 5B also shows fraction 6 with an intermediate composition between fractions 3 and 7. The composition of the peak in fraction 7 may reflect some contamination with early endosomes, because these are found at the same density. However, although reminiscent of fraction 7, the composition of early endosome fractions is distinct (PC ~50% and PE ~20% of total PLs) (13). Finally, fractions 11 and 12 (\(d = 1.1545–1.1654 \text{ g/cm}^3\)) contained primarily LBPA (~60%), PC (~20%), some PE (~10%), and low (<5%) levels of other PLs.

**Lamp1 and CD63**—We then analyzed the distribution of two late endosomal proteins, Lamp1 and CD63, which colocalize with LBPA in late endosomes (13, 30). In contrast to LBPA, the bulk of Lamp1 is present on the organelle-limiting membrane, even though the protein can occasionally be detected within internal membranes (13, 16, 32). After suborganellar fractionation, small amounts of Lamp1 (~15%) were found in fraction 12 containing LBPA, perhaps corresponding to some Lamp1 molecules present within LBPA-rich membranes. The bulk of Lamp1, however, peaked in fraction 7 with a complex PL composition (Fig. 7A) and did not co-fractionate with LBPA, indicating that Lamp1- and LBPA-containing membranes could be separated after late endosome subfractionation.
The tetraspanin CD63 is abundant within internal membranes, in marked contrast to Lamp1, but is also present on the organelle-limiting membrane (33, 34) (Fig. 8 B). Consistently, the protein distributes not only to vesicles but also to highly dynamic late endosomal tubules visualized by time-lapse video microscopy, like Lamp1 (35). We have shown recently (35) that both endogenous CD63 and green fluorescent protein (GFP)-tagged CD63 colocalize with both Lamp1 and LBPA in several cell types, including HeLa and BHK cells. We then analyzed the distribution of transiently expressed CD63-GFP by electron microscopy in HeLa cells because of their very high transfection rate. CD63-GFP distributed to perinuclear late endosomes (35) (Fig. 8 A) with a characteristic multivesicular ultrastructure (Fig. 8, C and D). Immunogold labeling of cryosections showed similar labeling patterns using anti-GFP antibodies (Fig. 8C) or anti-CD63 antibodies (Fig. 8D), which reveal both endogenous CD63 and CD63-GFP in these cells. CD63-GFP was abundant within internal membranes and also present on the limiting membrane, much like endogenous CD63 (33, 34) (Fig. 8B).

We therefore used CD63-GFP in BHK cells in our experiments, because existing anti-CD63 antibodies do not cross-react with BHK cells used in these fractionation studies. The overall distribution of CD63 was different from that of Lamp1 after subfractionation (Fig. 7B), because the bulk (~65%) of CD63-GFP was found in fractions 3–5 (containing mostly PC) and 12 (containing mostly LBPA) (Fig. 7B). CD63 (~30%) was also found in fractions 7 and 8 that contained Lamp1, presumably reflecting the presence of CD63 within both the limiting membrane and late endosomal tubules visualized by time-lapse...
video microscopy (35). Because the bulk of Lamp1 is present in the late endosome limiting membrane in vivo, and in fractions 6–8 after separation in our gradients, we conclude that the two membrane populations containing CD63 and either PC or LBPA, but not Lamp1, are likely to be derived from internal membranes.

**Endocytosis of the VSV-G Glycoprotein**—Internal membranes of multivesicular endosomes presumably contain proteins destined for degradation in lysosomes (36). To characterize further the membrane populations obtained after subfractionation, we used the trans-membrane glycoprotein G of VSV as marker of the degradation pathway. VSV-G can be incorporated into the plasma membrane by low pH-mediated fusion of the viral envelope with the plasma membrane (17–19). After cross-linking with antibodies, VSV-G is efficiently endocytosed and then degraded in lysosomes (18, 19). Fig. 9 shows that endocytosed VSV-G was transported to LBPA-positive late endosomes and that transport was inhibited by microtubule depolymerization (Fig. 9), as expected (19).

Late endosomes were then analyzed as above, after internalization of cross-linked VSV-G in the medium to limit degradation (Fig. 10B). VSV-G distributed across the entire gradient, with the bulk (~70%) in fractions 2–4 (d = 1.0388 to 1.0655 g/cm³) and 10–12 (d = 1.1423 to 1.1654 g/cm³). VSV-G was also present in fractions 7 and 8, containing Lamp1, consistently with its presence at the limiting membrane (18, 19). The broader VSV-G distribution, when compared with that of Lamp1, cannot be accounted for by differences in the amounts of the two proteins; very low amounts of VSV-G were incorporated into the plasma membrane (<1% of total plasma membrane protein) (18), whereas lysosomal glycoproteins are very abundant (~50% of total late endosomal/lysosomal proteins) (32). Interestingly, despite the presence of leupeptin, top and bottom fractions contained processed, trans-membrane forms of VSV-G, revealed using the P5D4 antibody against G-protein cytoplasmic domain (Fig. 10B). When using intact late endosomes as a control, VSV-G was recovered as a single peak (d = 1.0772 g/cm³) at the late endosome density (not shown), as expected (Fig. 2C). Similarly, when early endosomal fractions were prepared 5 min after VSV-G internalization (18, 19), VSV-G was also recovered as a single peak around fraction 7 (d = 1.1031 g/cm³), whether early endosomes had been subjected to the same freeze/thaw cycles...
Late Endosomal Membrane Domains

(Fig. 10A) or not (not shown). These experiments thus show that a cargo protein destined for degradation distributed within all membrane populations separated on our gradient, including those containing markers of internal membrane domains.

**DISCUSSION**

Our observations show that different membrane populations with a defined lipid composition can be physically separated after late endosome rupture under conditions sufficiently gentle to preserve early endosome integrity. It is unlikely that this heterogeneity simply reflects selective lipid partitioning during endosome rupture by freezing and thawing, because the same distribution was also observed after osmotic shock. In addition, this heterogeneity cannot be accounted for by the presence of early endosomes, recycling endosomes, or biosynthetic organelles in the initial fraction used as starting material. BHK late endosomes are recovered at a very low buoyant density in sucrose (∼1.0772 g/cm³), well separated from these organelles (13, 15, 16). More importantly, LBPA itself provides a convenient late endosome lipid marker, and all proteins we studied colocalize with LBPA in late endosomes (13, 30) (Fig. 7).

Lamp1, which is restricted to late endosome limiting membrane by immunogold labeling of cryosections, is found mostly in fractions with a complex lipid composition and yet distinct from that of early endosomes. This composition presumably reflects the fact that the limiting membrane is directly connected by membrane traffic pathways to other compartments. Late endosomes also form highly dynamic tubular regions that contain CD63 and Lamp1 (35), and these tubules may well account for a significant portion of the membrane population containing both proteins (fractions 7 and 8). In contrast, internal membranes form a highly specialized and privileged environment. As opposed to other vacuolar membranes, internal membranes are not directly integrated into the vacuolar membrane flow, a situation reminiscent of chloroplast and mitochondria inner membrane systems.

Suborganellar fractionation reveals the presence of two membrane populations, which are both well separated from Lamp1 membranes, suggesting that they originate from intraluminal membranes. Both populations contain CD63, which is predominantly present in internal membranes (33), and transmembrane processed forms of VSV-G, a marker of the degradation pathway (18). However, only one of these populations contains the bulk of LBPA, and LBPA is dramatically enriched in these membranes (∼70% of total PLs), whereas the second population is enriched in PC. This may seem surprising. However, internal membranes seem to be highly heterogeneous when visualized by electron microscopy, because late endosomes appear multivesicular or multilamellar, sometimes with highly ordered membrane arrays (37). Essentially nothing is known about the biochemical or biophysical nature of these differences. These membranes are also heterogeneous in composition, because LBPA is abundant in late endosomes but not in multivesicular intermediates at earlier stages of the pathway (13). In addition, LBPA, although present in significant amounts, represents only ∼15% of the total PLs in the late endosomal fraction. LBPA is thus unlikely to be the single major PL species of internal membranes, because these are very abundant in multivesicular regions (16, 32). In contrast, PC is the most abundant lipid (45% of total lipids; 9:1 molar ratio PC:LBPA) and, as such, should represent a major constituent of internal membranes.

In addition to morphological and compositional differences, internal membranes also appear to fulfill different functions. They accumulate molecules destined to be degraded (19, 36), consistently with the findings that LBPA and other negatively charged PLs facilitate glycolipid degradation (38). However, internal membranes also accumulate Man-6-P receptor in transit (32), tetraspanins (33), and major histocompatibility complex class II receptor (39). In fact, the cycle of Man-6-P receptor is inhibited when interfering with LBPA functions, as is cholesterol transport (13, 40). Conversely, cholesterol accumulation inhibits Man-6-P receptor and CD63 transport (30, 34).

Finally, our observations show that LBPA membranes separated on the gradient contain CD63 but also processed forms of VSV-G. It thus appears that LBPA membranes have a dual role in degradation and transport, perhaps suggesting that these membranes have turnpike functions within the complex system of late endosomal internal membranes. It is attractive to believe that late endosome internal membranes may be composed primarily of PC, a lipid with a preference for fluid regions that can accommodate the high curvature of internal vesicles and tubules but that the organization and dynamic properties of these membranes depend on LBPA domains. Indeed, the cone-shaped structure and pH-dependent fusion activity of LBPA make it an ideal candidate to regulate internal membrane biogenesis and interactions. We conclude that late endosome inner membranes form a mosaic of lipid domains and that their dynamic properties depend on LBPA.

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