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Identification of the phospholipid lysobisphosphatidic acid in the protozoan *Entamoeba histolytica*: An active molecule in endocytosis

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ABSTRACT

Phospholipids are essential for vesicle fusion and fission and both are fundamental events for *Entamoeba histolytica* phagocytosis. Our aim was to identify the lysobisphosphatidic acid (LBPA) in trophozoites and investigate its cellular fate during endocytosis. LBPA was detected by TLC in a 0.5 R_f spot of total lipids, which co-migrated with the LBPA standard. The 6C4 antibody, against LBPA recognized phospholipids extracted from this spot. Reverse phase LC-ESI-MS and MS/MS mass spectrometry revealed six LBPA species of *m*/*z* 772.58–802.68. LBPA was associated to pinosomes and phagosomes. Intriguingly, during pinocytosis, whole cell fluorescence quantification showed that LBPA dropped 84% after 15 min incubation with FITC-Dextran, and after 60 min, it increased at levels close to steady state conditions. Similarly, during erythrophagocytosis, after 15 min, LBPA also dropped in 36% and increased after 60 and 90 min. EhRab7A protein appeared in some vesicles with LBPA in steady state conditions, but after phagocytosis co-localization of both molecules increased and in late phases of erythrophagocytosis they were found in huge phagosomes or multivesicular bodies with many intraluminal vacuoles, and surrounding ingested erythrocytes and phagosomes. The 6C4 and anti-EhADH (EhADH is an ALIX family protein) antibodies and Lysotracker merged in about 50% of the vesicles in steady state conditions and throughout phagocytosis. LBPA and EhADH were also inside huge phagosomes. These results demonstrated that *E. histolytica* LBPA is associated to pinosomes and phagosomes during endocytosis and suggested differences of LBPA requirements during pinocytosis and phagocytosis.

1. Introduction

Entamoeba histolytica is the protozoan causative agent of human amoebiasis. It affects 50 million people around the world producing dysentery and liver abscesses [1]. Trophozoites are professional phagocytes and constitute the mobile and invasive phase of the parasite. Several proteins participating in phagocytosis have been identified, among them the Gal/GalNac lectin [2], EhC2PK, EhCaBP1, EhAK1 [3,4], several EhRab proteins [5–9] and the EhCPADH complex [10]. EhCPADH is formed by a protease (EhCP112) and an adhesin (EhADH) [10], a member of the ALIX family [11,12]. Lipids also influence the endosome membrane properties by changing biophysical characteristics and by recruiting proteins involved in membrane remodeling [13]. In addition, they protect trophozoites from the huge amount of endogenous proteases and amoebapore-forming proteins [14]. It has been reported that phosphoinositides are involved in the phagocytic cup formation, but not in the initial host cell interaction, neither at intermediate and late phases of phagocytosis and nor during pinocytosis [15,16]; though, earlier publications suggested that PI3-kinase inhibitors, diminish pinocytosis and parasite-host adherence [17]. Cholesterol is not synthesized by the parasite, even when it is essential for virulence expression [17,18]. Another intriguingly fact is that trophozoites have a higher ceramide proportion in comparison with mammalian cells [13,19]. However, the biological significance of this has not been fully elucidated.

In eukaryotes, plasma membrane invagination to trap the prey or cargo molecules is followed by endosomes and multivesicular bodies (MVBs) formation. In MVBs, some intraluminal vesicles (ILVs), carrying cargo molecules, are fused to other vesicles and lysosomes; whereas, vesicles carrying receptors are recycled to plasma membrane and other organelles [20]. Throughout

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maturation, endosomes modify pH, size, appearance and protein and lipids content [21,22]. The endosomal-sorting complex required for transport (ESCRT) and its accessory proteins, Alix and Vps4 ATPasa [23,24], participate in endocytosis. In addition, PI3P [25], PI(3,5)P_2 [26], cholesterol [27] and the phospholipid lysobisphosphatidic acid (LBPA), also named bis(monoacyl)glycerolphosphate(BMP) confer to the membranes specific characteristics to be remodeled during endocytosis [28,29].

Functional LPBA presents one fatty acid chain attached to the C2 of the two-glycerol backbones [30,31] and in general, its proportion of polyunsaturated acyl chains is higher than in other phospholipids [32–34]. LBPA is found mainly in acidic vesicles with high hydrolases content [35,36] and it is highly resistant to lipases and phospholipases. LBPA is present in animal tissues in a small amount, but it is enriched in vesicles inside late endosomes [37–39]. Using BHK cell membranes of late endosomes, Kobayashi et al. [38] generated a monoclonal antibody (6C4) against LBPA. LBPA is associated with Rab7, and interacts Alix, Niemann-Pick C (NPC) and saposin-C proteins during endocytosis. It participates in cholesterol distribution and homeostasis [28,37,38,40,41], sphingolipid metabolism [42], viral infection [43] and autoimmune diseases. Thus, LBPA is a critical component of endosomal/lysosomal network and it is essential for MVBs formation.

LBPA had not been identified in *E. histolytica* trophozoites. Here, we used the 6C4 antibody, reverse phase HPLC coupled to electrospray ionization mass spectrometry (ESI-MS) and tandem mass spectrometry (MS/MS) techniques, to reveal LBPA as a component of its phospholipid fraction. Our results demonstrated that LBPA is in endosomes during dextran uptake and erythrophagocytosis and it appeared associated to EhRab7A and EhADH proteins.

2. Materials and methods

2.1. Reference standards

(*S*,*S*)-2,2´-bisoleoyl-LBPA phospholipid standard was purchased from Echelon Bioscience in its lyophilized tetrabutylammonium salt.

2.2. Reagents

Dextran and FITC-dextran (mol wt 70,000) were from Sigma–Aldrich. Solvents for high performance liquid chromatography (HPLC) water (ChromAR®) and n-hexane (UltimAR®) were obtained from Macron Fine Chemicals. Anti-LBPA monoclonal antibodies (6C4 supernatant) were purchased from Echelon Bioscience. Secondary antibodies were purchased from Zymed and Invitrogen; anti-EhADH antibodies were generated in our group by immunizing rabbits twice each two weeks with 120 μg of a polypeptide corresponding to the EhADH C-terminus (566-QCVINLLKEFDNTKNI-582) coupled to the carrier protein Keyhole limpet hemocyanin (KLH), using TiterMax Classical Adjuvant (1:1 v/v) (Sigma-Aldrich). Anti-EhRab7A antibodies were kindly given by Dr. Tomoyoshi Nozaki [7].

2.3. E. histolytica cultures

Trophozoites of *E. histolytica* (strain HM1:IMSS) were axenically cultured in TYI-S-33 medium at 37 °C and harvested after 72 h [44]. Cell viability was monitored by optical microscopy and using Trypan blue dye exclusion test. Experiments presented here were performed at least three times in duplicate.

2.4. Lipids extraction procedure

Total lipids were extracted according to Folch [45]. Briefly, 120×10^6 trophozoites were placed in an extraction vial with 5 mL of methanol and incubated 20 min at 55 °C. Then, 2 volumes of chloroform were added and after sonication and vortexing, samples were incubated overnight (ON) at room temperature (RT). Samples were vortexed again, centrifuged for 10 min at 866*g* and filtered through a disc filter Whatman 1 M. Organic layer was collected, dried under liquid nitrogen and stored at −20 °C. An aliquot of total lipid extracts was used to determine phospholipids content [46].

2.5. Enzyme linked immunoassays (ELISA)

Wells of microtiter plates were coated with 0, 40 or 100 μg of trophozoites lipid extract dissolved in 100 μl of methanol:chloroform (98:2% v/v) and evaporated at RT. As a negative control, we added to other wells 40 or 100 μg of trophozoite proteins. As a positive control we employed 10 μg of (*S*,*S*)-2,2´-bisoleoyl-LBPA standard. Samples were blocked with 10% fetal bovine serum (FBS) in PBS and then, 10 μg/mL of 6C4 antibody (1:50) in PBS were added to the wells, which then, were incubated for 90 min at RT. Antibody was detected by anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (1:3000), incubated for 1 h at RT and developed by *O*-phenylendiamine substrate (Zymed). Optical density (OD_{492}) was measured in a spectrophotometer (iMark, Biorad).

2.6. Thin layer chromatography (TLC)

Trophozoites lipid extracts were solubilized and spotted on TLC silica plates (Merck). Plates were developed by *n*-hexane:isopropanol:water (12:16:3 v/v/v) at RT for 6 h. Phospholipids were revealed by iodine vapors and they were identified by comparison with (*S*,*S*)-2,2´-bisoleoyl-LBPA standard spotted on the same silica plate. Solvent was removed using nitrogen flow.

2.7. Dot blot assays

Phospholipid fractions separated by TLC were scrapped off from the silica and dissolved in isopropanol/water (95:5%). Then, 50 μg of each one of the lipids extracted from the silica, 50 μg of LBPA and 50 μg of total lipid extracts from trophozoites were spotted on a polyvinylidene difluoride (PVDF) membranes. As a negative control we used lecithin and the secondary antibody on total lipids. Lipid spots were dried and filters were blocked with 10% FBS in PBS, ON at 4 °C. Membranes were then incubated for 3 h at RT with 6C4 antibody (1:100), washed four times with PBS-Tween 20, (0.02%) and revealed with HRP-conjugated anti-mouse antibody (1:9000). After washing several times with PBS-Tween 20, reactivity was visualized using a commercial enhanced chemiluminescence imaging system MicroChem 4.2 (Bio Imaging System).

2.8. High-performance liquid chromatography (HPLC) analysis

Samples of total lipids were dissolved in methanol. Reverse-phase HPLC was performed according to Mortuza et al. [34] using an Agilent 1 200 capillary LC pump chromatograph. Phospholipid elution was carried out using a binary system as follows: Eluent A: 0.25% (v/v) ammonium hydroxide/0.05% (v/v) formic acid in methanol, pH 6.4:water (88:12). Eluent B: 0.25% (v/v) ammonium hydroxide/0.05% (v/v) formic acid in methanol, pH 6.4:hexane (80:20). All mobile phases were freshly prepared, filtered through 0.22 μm filter (Millipore) and degassed under vacuum. Samples