A novel imaging method revealed phosphatidylinositol 3,5-bisphosphate-rich domains in the endosome/lysosome membrane

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\textbf{ABSTRACT}

We developed a new method to observe distribution of phosphatidylinositol 3,5-bisphosphate [PtdIns(3,5)P\textsubscript{2}] using electron microscopy. In freeze-fracture replicas of quick-frozen samples, PtdIns(3,5)P\textsubscript{2} was labeled specifically using recombinant ATG18 tagged with glutathione S-transferase and 4×FLAG, which was mixed with an excess of recombinant PX domain to suppress binding of ATG18 to phosphatidylinositol 3-phosphate. Using this method, PtdIns(3,5)P\textsubscript{2} was found to be enriched in limited domains in the yeast vacuole and mammalian endosomes. In the yeast vacuole exposed to hyperosmolar stress, PtdIns(3,5)P\textsubscript{2} was distributed at a significantly higher density in the intramembrane particle (IMP)-deficient liquid-ordered domains than in the surrounding IMP-rich domains. In mammalian cells, PtdIns(3,5)P\textsubscript{2} was observed in endosomes of tubulo-vesicular morphology labeled for Rab5 or Rab7. Notably, distribution density of PtdIns(3,5)P\textsubscript{2} in the endosome was significantly higher in the vesicular portion than in the tubular portion. The nanoscale distribution of PtdIns(3,5)P\textsubscript{2} revealed in the present study is important to understand its functional roles in the vacuole and endosomes.


deficiency is linked to diseases such as Charcot-Marie-Tooth disease and amyotrophic lateral sclerosis.\textsuperscript{4,6}

To further understand the physiological function of PtdIns(3,5)P\textsubscript{2}, it is important to know its distribution in detail. We, thus, developed a new electron microscopic method to visualize the nano-scale distribution of PtdIns(3,5)P\textsubscript{2} in a semi-quantitative manner and found for the first time that PtdIns(3,5)P\textsubscript{2}-rich and -deficient membrane domains coexist both in the yeast vacuole and in mammalian endosomes.

Phosphatidylinositol 3,5-bisphosphate [PtdIns(3,5)P\textsubscript{2}] is a least abundant phosphoinositide, but it is thought to exert critical functions mainly in the endosome and lysosome.\textsuperscript{1-3} PtdIns(3,5)P\textsubscript{2} deficiency is linked to diseases such as Charcot-Marie-Tooth disease and amyotrophic lateral sclerosis.\textsuperscript{4,6}

The above four difficulties could be overcome or avoided using the quick-freezing and freeze-fracture replica labeling method (QF-FRL).\textsuperscript{8,11} (Fig. 1A). First, in QF-FRL, membrane lipids are physically fixed without chemical fixatives, by freezing and then by vacuum
evaporation of carbon and platinum. Second, cells without any pretreatment are used in QF-FRL. Third, the labeling specificity can be examined by QF-FRL per se using freeze-fracture replicas of liposomes containing different lipids. Fourth, the probe used to label PtdIns(3,5)P2 (recombinant ATG18 tagged with glutathione S-transferase (GST) and 4\(^\times\)FLAG [GST-ATG18-4\(^\times\)FLAG]) showed virtually no binding to PtdIns(5)P. A minimal but non-negligible amount of GST-ATG18-4\(^\times\)FLAG binding with PtdIns(3)P could be eliminated by mixing an excessive amount of recombinant tag-free p40\(^{phos}\) PX domain, which specifically binds to PtdIns(3)P (Fig. 1B).

Using the QF-FRL method, we examined distribution of PtdIns(3,5)P2 in budding yeast under hyperosmotic stress and in mammalian culture cells. In yeast exposed to hyperosmosis, vacuoles undergo fragmentation by a PtdIns(3,5)P2-dependent mechanism.\(^{14,15}\) In the vacuolar membrane under the hyperosmotic stress, we found formation of IMP-deficient domains, where PtdIns(3,5)P2 is enriched compared to surrounding IMP-rich domains. The IMP-deficient domain is thought to represent a liquid-ordered phase because VPH1, a V0 component of V-ATPase and a marker of liquid-disordered phase,\(^{16}\) was not present in this domain. In yeast that is deficient in PtdIns(3,5)P2 synthesis or ATG18, a putative PtdIns(3,5)P2 effector, the IMP-deficient domain formed aberrant double-walled tubular structures in the vacular lumen. This indicates that proper generation of the PtdIns(3,5)P2-rich domain is critical for the normal vacuole fragmentation process.

On the other hand, in mammalian cells, PtdIns(3,5)P2 was observed in tubulo-vesicular endosomes that were labeled for either RAB5 or RAB7. Notably, the PtdIns(3,5)P2 label was found in a significantly higher density in the vesicular portion (pink) than in the tubular portion (green).
In both the yeast vacuole and mammalian endosomes, biased distribution of PtdIns(3,5)P$_2$ within the membrane should have functional importance by recruiting specific effectors. In the yeast vacuole, ATG18 is likely to play a major role in vacuole fragmentation in the PtdIns(3,5)P$_2$-rich domain. In mammalian endosomes, enrichment of PtdIns(3,5)P$_2$ in the vesicular portion is thought to be relevant for endosome functionality by activating ion channels and inducing formation of intraluminal vesicles. On the other hand, the relative paucity of PtdIns(3,5)P$_2$ in the tubular portion suggests that PtdIns(3,5)P$_2$ plays only a minor role in binding of the retromer complex.18,19

We expect that defining the nano-scale PtdIns(3,5)P$_2$ distribution will provide new information on the functionality of the vacuole and the endosome/lysosome, and on lipid domains in those membranes.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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