COMMUNICATIVE & INTEGRATIVE BIOLOGY
2016, VOL. 9, NO. 2, e1145319 (4 pages)
http://dx.doi.org/10.1080/19420889.2016.1145319

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ARTICLE ADDENDUM

OPEN ACCESS

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

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ABSTRACT

We developed a new method to observe distribution of phosphatidylinositol 3,5-bisphosphate [PtdIns(3,5)P_2] using electron microscopy. In freeze-fracture replicas of quick-frozen samples, PtdIns(3,5)P_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_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_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_2 was observed in endosomes of tubulo-vesicular morphology labeled for RAB5 or RAB7. Notably, distribution density of PtdIns(3,5)P_2 in the endosome was significantly higher in the vesicular portion than in the tubular portion. The nano-scale distribution of PtdIns(3,5)P_2 revealed in the present study is important to understand its functional roles in the vacuole and endosomes.

ARTICLE HISTORY
Received 13 January 2016
Accepted 18 January 2016

KEYWORDS
electron microscopy; endosome; freeze-fracture; hyperosmotic stress; lysosome; membrane domain; phosphatidylinositol 3,5-bisphosphate; vacuole

Phosphatidylinositol 3,5-bisphosphate [PtdIns(3,5)P_2] is a least abundant phosphoinositide, but it is thought to exert critical functions mainly in the endosome and lysosome.[1-3] PtdIns(3,5)P_2 deficiency is linked to diseases such as Charcot-Marie-Tooth disease and amyotrophic lateral sclerosis.[4-6]

To further understand the physiological function of PtdIns(3,5)P_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_2 in a semi-quantitative manner and found for the first time that PtdIns(3,5)P_2-rich and -deficient membrane domains coexist both in the yeast vacuole and in mammalian endosomes.

Defining membrane lipid distribution at a small scale is challenging for several reasons.[7] First, because most lipids do not react with aldehydes that are commonly used as a fixative, morphological methods used for proteins may give artifactual results for lipids.[8,9] Second, GFP technology can be applied to lipid studies only indirectly by expressing GFP-tagged lipid-binding proteins,[10] but their expression in living cells may perturb functionality of the target lipids. Third, binding specificity of probes for lipids may vary depending on the assay method; for example, a protein probe binding to a specific lipid in a biochemical assay might behave differently in an imaging method.[11] Fourth, PtdIns(3,5)P_2-binding probes could also bind to phosphatidylinositol 3-phosphate [PtdIns(3)P] and/or phosphatidylinositol 5-phosphate [PtdIns(5)P], which are more abundant than PtdIns(3,5)P_2 and are likely to distribute near PtdIns(3,5)P_2. Cross-reactivity to PtdIns(5)P is particularly difficult to assess in a cellular context because inhibition of PtdIns(3,5)P_2 synthesis also decreases the amount of PtdIns(5)P.[12]

The above four difficulties could be overcome or avoided using the quick-freezing and freeze-fracture replica labeling method (QF-FRL)[9,13] (Fig. 1A). First, in QF-FRL, membrane lipids are physically fixed without chemical fixatives, by freezing and then by vacuum

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Color versions of one or more of the figures in the article can be found online at www.tandfonline.com/kcib.
Addendum to: Takatori S, Tatematsu T, Cheng J, Matsumoto J, Akano T, Fujimoto T. Phosphatidylinositol 3,5-bisphosphate-rich membrane domains in endosomes and lysosomes. Traffic 2015; http://dx.doi.org/10.1111/tra.12346

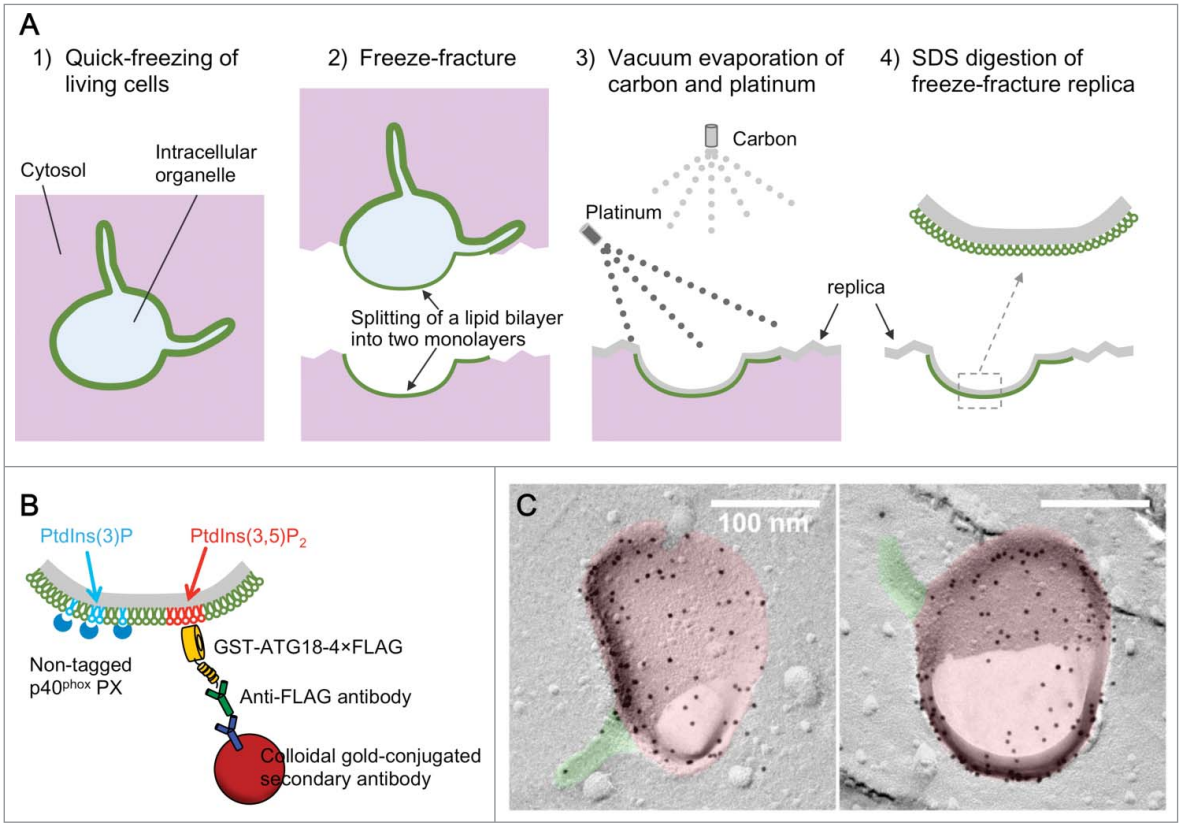

Figure 1. (A) QF-FRL procedure. 1) Quick-freezing stops molecular motion instantaneously. 2) Freeze-fracture splits membranes into two phospholipid monolayers. 3) Vacuum evaporation of carbon and platinum coats the phospholipid monolayer from the hydrophobic side, thus physically stabilizing the structure. 4) SDS treatment removes extramembrane materials and exposes the hydrophilic membrane surface. (B) The combination of probes that labeled PtdIns(3,5)P_2 in QF-FRL. GST-ATG18-4×FLAG bound to PtdIns(3,5)P_2 is visualized by colloidal gold under EM. An excess non-tagged $p40^{phox}$ PX domain blocks binding of GST-ATG18-4×FLAG to PtdIns(3)P. (C) PtdIns(3,5)P_2 in the tubulo-vesicular endosome of HeLa cells. The label was found in a significantly higher densely in the vesicular portion (pink) than in the tubular portion (green).

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)P_2 (recombinant ATG18 tagged with glutathione S-transferase (GST) and 4×FLAG [GST-ATG18-4×FLAG]) showed virtually no binding to PtdIns(5)P. A minimal but non-negligible amount of GST-ATG18-4×FLAG binding with PtdIns(3)P could be eliminated by mixing an excessive amount of recombinant tag-free $p40^{phox}$ PX domain, which specifically binds to PtdIns(3)P[9] (Fig. 1B).

Using the QF-FRL method, we examined distribution of PtdIns(3,5)P_2 in budding yeast under hyperosmotic stress and in mammalian culture cells. In yeast exposed to hyperosmosis, vacuoles undergo fragmentation by a PtdIns(3,5)P_2-dependent mechanism.[14,15] In the vacuolar membrane under the hyperosmolar stress, we found formation of IMP-deficient domains, where PtdIns(3,5)P_2 is enriched compared to surrounding IMP-rich domains. The IMP-deficient domain is thought to represent a liquid-ordered phase because VPH1, a V_0 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)P_2 synthesis or ATG18, a putative PtdIns(3,5)P_2 effector, the IMP-deficient domain formed aberrant double-walled tubular structures in the vacuolar lumen. This indicates that proper generation of the PtdIns(3,5)P_2-rich domain is critical for the normal vacuole fragmentation process.

On the other hand, in mammalian cells, PtdIns(3,5)P_2 was observed in tubulo-vesicular endosomes that were labeled for either RAB5 or RAB7. Notably, the PtdIns(3,5)P_2 label was found in a significantly higher density in the vesicular portion than in the tubular portion of the endosomes (Fig. 1C). The relative enrichment of PtdIns(3,5)P_2 in the vesicular portion of endosomes was also observed by fluorescence microscopy using a biosensor for PtdIns(3,5)P_2.[17] In contrast, VPS35, a retromer component, was in the tubular portion and segregated from PtdIns(3,5)P_2.

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.[1,2] 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.

Acknowledgments

We are grateful to our lab members for helpful discussions. ST was a research fellow of the Japan Society for the Promotion of Science (JSPS).

Funding

This work was supported by a Grant-in-Aid for JSPS Fellows, a Grant-in-Aid for Young Scientists (B) from JSPS (to ST), a Grant-in-Aid for Scientific Research and a Grant-in-Aid for Scientific Research on Innovative Areas (to TF).

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