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Research paper

Nanoscale domain formation of phosphatidylinositol 4-phosphate in the plasma and vacuolar membranes of living yeast cells

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A B S T R A C T

In budding yeast *Saccharomyces cerevisiae*, PtdIns(4)P serves as an essential signalling molecule in the Golgi complex, endosomal system, and plasma membrane, where it is involved in the control of multiple cellular functions via direct interactions with PtdIns(4)P-binding proteins. To analyse the distribution of PtdIns(4)P in yeast cells at a nanoscale level, we employed an electron microscopy technique that specifically labels PtdIns(4)P on the freeze-fracture replica of the yeast membrane. This method minimizes the possibility of artificial perturbation, because molecules in the membrane are physically immobilised *in situ*. We observed that PtdIns(4)P is localised on the cytoplasmic leaflet, but not the exoplasmic leaflet, of the plasma membrane, Golgi body, vacuole, and vesicular structure membranes. PtdIns(4)P labelling was not observed in the membrane of the endoplasmic reticulum, and in the outer and inner membranes of the nuclear envelope or mitochondria. PtdIns(4)P forms clusters of < 100 nm in diameter in the plasma membrane and vacuolar membrane according to point pattern analysis of immunogold labelling. There are three kinds of compartments in the cytoplasmic leaflet of the plasma membrane. In the present study, we showed that PtdIns(4)P is specifically localised in the flat undifferentiated plasma membrane compartment. In the vacuolar membrane, PtdIns(4)P was concentrated in intramembrane particle (IMP)-deficient raft-like domains, which are tightly bound to lipid droplets, but not surrounding IMP-rich non-raft domains in geometrical IMP-distributed patterns in the stationary phase. This is the first report showing microdomain formations of PtdIns(4)P in the plasma membrane and vacuolar membrane of budding yeast cells at a nanoscale level, which will illuminate the functionality of PtdIns(4)P in each membrane.

1. Introduction

Phosphoinositides (PPIn) are essential phospholipids that serve as key regulators of numerous cellular processes, although they are minor constituents of cellular membranes, representing about 1% of total cellular phospholipids (Payrastre et al., 2001; Balla, 2013). Phosphatidylinositol 4-phosphate (PtdIns(4)P) accounts for about 30% of total PPIns in yeast, and approximately 45% in humans (Payrastre et al., 2001). PtdIns(4)P is converted from PtdIns, PtdIns(4,5)P_2, and PtdIns (3,4)P_2 by PtdIns-4 kinase, PPIn 5-phosphatase, and PPIn 3-phosphatase, respectively. Defects in PtdIns(4)P signalling are linked to diseases such as Cowden's disease and cancer for PPIn 3-phosphatase PTEN (Leslie and Longy, 2016), Lowe syndrome for PPIn 5-phosphatase OCRL1 (Lin et al., 1997), Joubert and MORM syndrome (two ciliopathies) for PPIn 5-phosphatase INPP5e (Bielas et al., 2009; Jacoby et al., 2009), and Parkinson's disease for PPIn 5-phosphatase Synaptojanin 1 (Krebs et al., 2013; Quadri et al., 2013), supporting the physiological importance of PtdIns(4)P.

PtdIns(4)P was originally thought to be the precursor of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2), which plays critical roles

Abbreviations: CSR, complete spatial randomness; EM, electron microscopy; FAPP1, four-phosphate-adaptor protein 1; FRAP, fluorescence recovery after photobleaching; GFP, green fluorescent protein; GST, glutathione *S*-transferase; IMP, intramembrane particle; INPP5E, inositol polyphosphate-5-phosphatase E; LD, lipid droplet; LTP, lipid transfer protein; MCC, membrane compartment containing Can1; MCP, membrane compartment containing Pma1; MCT, membrane compartment containing TORC2; MORM, mental retardation, truncal obesity, retinal dystrophy, and micropenis; OCRL, oculo-cerebro-renal-Lowe; OSBP, oxysterol-binding protein; PH, pleckstrin homology; PPIn, phosphoinositides; PLC, phospholipase C; PtdIns, phosphatidylinositol; PTEN, phosphatase and tensin homolog deleted from chromosome 10; QR-FRL, quick-freezing and freeze-fracture replica labelling; SDS, sodium dodecylsulfate

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in multiple cellular functions. However, PtdIns(4)P itself has recently been found to serve as an essential signalling molecule in the Golgi apparatus, endosomal system, and plasma membrane, where it is involved in the control of membrane trafficking, cytoskeletal organization, lipid metabolism, lipid transport, and signal transduction pathways via direct interaction with PtdIns(4)P-binding proteins (D'Angelo et al., 2008; Hammond et al., 2012; Mesmin and Antonny, 2016; Yoshida et al., 2016). Because the abundance of PtdIns(4)P in the plasma and organelle membranes is critical for many cellular functions, understanding PtdIns(4)P dynamics in the plasma membrane and intracellular organelle membranes is essential (Yoshida et al., 2016; Yoshida et al., 2017). The budding yeast *Saccharomyces cerevisiae* has proven useful as a genetic model system to study PPIn signalling pathways, because several of the lipid kinase, lipid phosphatases, and effectors are conserved (Odorizzi et al., 2000). In yeast, PtdIns(4)P is mainly present in two distinct compartments: the plasma membrane and the Golgi body (Audhya et al., 2000; De Craene et al., 2017). PtdIns (4)P performs functions in membrane trafficking from the Golgi body to the plasma membrane and the intracellular organelles (Audhya et al., 2000; Godi et al., 2004; Mizuno-Yamasaki et al., 2010), which requires tight spatiotemporal regulation of PtdIns(4)P levels at each membrane site. To further understand the exact functions of PtdIns(4)P, it is critically important to study its subcellular distribution in the yeast membrane at a nanoscale level.

In the present study, we employed quick-freezing and freeze-fracture replica labelling (QF-FRL) electron microscopy (EM) (Fujita et al., 2010). QF-FRL involves quick-freezing of cells, followed by physical stabilisation of the membranes by freeze-fracture replica formation, i.e. vacuum evaporation of platinum and carbon (Fig. 1A). Most lipids are not reactive with conventional chemical fixatives such as aldehyde, and so they may be dislodged from native locations giving rise to artefactual results in aldehyde fixed specimens (Heffer-Lauc et al., 2005). In contrast, using QF-FRL, lipids are captured *in situ* and physically immobilised in the freeze-fracture replica. QF-FRL also differs from other methods because it does not require the expression of binding probes in live cells, which might also perturb the distribution of endogenous target lipids (Takatori et al., 2014). Other limitations of the GFP-tagged domain method have been previously described (Irvine, 2004; Downes et al., 2005; Balla, 2007), and include: low spatial resolution, dramatic changes in Golgi structure and function upon expression of a GFP-PtdIns(4)P-binding domain probe (Levine and Munro, 1998; Balla et al., 2005), and the low affinity of the GFP-PtdIns(4)P-binding domain probe for PtdIns(4)P that is bound to endogenous proteins. Furthermore, yeast cell organelles are not sufficiently large and distant from each other to be solved at a light microscopic level, unlike mammalian cells. Because of these advantages, QF-FRL is thought to be one of the few methods that can define the two-dimensional distribution of membrane lipids at the nanoscale level (Fujita et al., 2010; Takatori et al., 2014).

In the present study, for the first time, we determined that PtdIns(4)P is localised on the cytoplasmic leaflet of the vacuole membrane under physiological conditions (Tahirovic et al., 2005). We also unexpectedly revealed that PtdIns(4)P forms cluster domains in the plasma membrane and vacuole membrane. The observed enrichment of PtdIns(4)P in each organelle is thought to be important for the proper functioning of the plasma membrane and intracellular organelles.

2. Materials and methods

2.1. Antibodies

The following antibodies were purchased from commercial sources: mouse monoclonal (IgM) anti-PtdIns(4)P antibody (Echelon Biosciences, Salt Lake City, UT, USA) and 10 nm gold particle-conjugated goat anti-mouse IgG + IgM antibody (EM.GAF 10; BBI Solutions, Cardiff, UK).

2.2. Probes

Recombinant GST fusion protein, containing the phospholipase C (PLC)-δ1 PH domain (GST-PPH), was expressed in *Escherichia coli* and purified as described previously (Yoshida et al., 2016). The pH domain of PLC-δ1 was obtained from the GST-pH fusion protein by cleavage with PreScission protease (GE Healthcare Life Sciences, Pittsburgh, PA, USA).

2.3. Yeast

Dr. Yoshinori Ohsumi of Tokyo Institute of Technology and Dr. Toyoshi Fujimoto of Nagoya University kindly provided the strain of *Saccharomyces cerevisiae* SEY6210 (*MATAα leu2-3, 112 ura3-52 his3-Δ200 trpl-Δ901 lys2-801 suc2Δ9 GAL)* used in the present study. The cells were grown in YPD medium (1% yeast extract, 2% Bacto-peptone and 2% dextrose) at 30 °C, and were harvested during the transition to exponential or stationary phase.

2.4. High pressure-freezing and freeze-fracture

Specimens were subjected to quick-freezing and freeze-fracture as described previously (Cheng et al., 2014). Briefly, a gold EM grid (200 mesh) impregnated with a small volume of yeast was sandwiched between two flat aluminium discs (3 mm diameter, 0.5 mm thick, Engineering Office M. Wholwend GmbH, Sennwald, Switzerland). The sample assembly was frozen using an HPM010 high-pressure freezing machine (Leica, Vienna, Austria), according to the manufacturer's instructions. The time required for freezing was ∼10 ms, which should be sufficiently fast to preserve PtdIns(4)P distribution *in situ*.

For freeze-fracture, the frozen cell sandwich was transferred to the cold stage of a Balzers BAF400 apparatus (Bal-Tec AG, Balzers, Lichtenstein) and fractured at −130 °C under a vacuum of $\sim 1 \times 10^{-6}$ millibar. Replicas were made by electron-beam evaporation in three steps: carbon (C) (2–5 nm in thickness) at an angle of 90° to the specimen surface, platinum-carbon (1–2 nm) at an angle of 45° and C (10–20 nm) at an angle of 90° (Fujita and Fujimoto, 2007; Fujita et al., 2010). The replica thickness of the deposition was adjusted by referring to a crystal thickness monitor (EM QSG100, Leica).

Thawed replicas were treated with 2.5% sodium dodecyl sulphate (SDS) in 0.1 M Tris-HCl (pH 8.0) at 60–70 °C overnight. To remove the cell wall attaching to yeast replicas, they were digested with 1 mg/ml Zymolyase 20T in phosphate buffered saline (PBS) containing 0.1% Triton X-100, 1% bovine serum albumin (BSA) and a protease inhibitor cocktail (Nacalai Tesque) for 2 h at 37 °C. After a further treatment in 2.5% SDS, the replicas were stored in buffered 50% glycerol at −20 °C until use.

2.5. Labelling and electron microscopy

Labelling with probes was performed as described previously (Fujita et al., 2010; Cheng et al., 2014; Yoshida et al., 2016). Briefly, freeze-fracture replicas were washed with PBS containing 1% Triton X-100 (PBST), then blocked with PBS containing 3% BSA at 25 °C for 30 min. For labelling PtdIns(4)P, replicas were pre-treated with the PH domain (1 mg/ml) of PLC-δ1 in blocking solution, to block nonspecific binding of the anti-PtdIns(4)P antibody to PtdIns(4,5)P_2 (Yoshida et al., 2016; Yoshida et al., 2017). Replicas were incubated with the anti-PtdIns(4)P mouse monoclonal primary antibody (1 μg/ml) diluted in PBS containing 1% BSA at 4 °C overnight. After four washes with PBS containing 0.1% BSA, replicas were incubated with 10 nm gold-conjugated anti-mouse IgG + IgM antibody (1/40 dilution) in PBS containing 1% BSA at 37 °C for 30 min. Replicas were placed on Formvar-coated grids and observed under a H7000KU electron microscope (HITACHI, Tokyo, Japan) operated at 75 kV.

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