主論文の要約

A new electron microscopic method to observe the distribution of phosphatidylinositol 3,4-bisphosphate

（ホスファチジルイノシトール 3, 4-二燐酸の局在観察のための新しい電子顕微鏡法）

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【Introduction】

The physiological importance of PtdIns(3,4)P_2 being revealed, the need to determine where and how PtdIns(3,4)P_2 is located in the cell is more urgent than ever. Although methods to quantify the amount of PtdIns(3,4)P_2 in isolated samples have been developed, considering the quick time course over which PtdIns(3,4)P_2 is thought to be generated and hydrolyzed, microscopic methods to observe its distribution are essential for understanding PtdIns(3,4)P_2 functions more in detail. In this context, the GFP (or YFP)-tagged pleckstrin homology (PH) domain of TAPP1 was used for live imaging, and recombinant TAPP1-PH and anti-PtdIns(3,4)P_2 antibody were used for labeling fixed cells and tissues. In the present study, we aimed to establish a method to observe the distribution of PtdIns(3,4)P_2 by the quick-freezing and freeze-fracture replica labeling (QF-FRL) technique. In this method, lipids are physically immobilized by freezing followed by freeze-fracture replica formation, and are then labeled for electron microscopic observation. We found that GST-tagged TAPP1-PH labels PtdIns(3,4)P_2 with a higher specificity than a commercially available anti-PtdIns(3,4)P_2 antibody in freeze-fracture replicas. The QF-FRL method using GST-tagged TAPP1-PH as the probe is expected to be useful in delineating the fine distribution of PtdIns(3,4)P_2 in various settings.

【Materials and Methods】

Cell culture and probes

Mouse NIH3T3 cells were serum-starved for 16 h in a medium containing 0.5% FBS before being treated with either 10 mM H_2O_2 or 5 ng/ml recombinant mouse platelet-derived growth factor. The PH domain of TAPP1 (amino acids 182–303) was amplified from HeLa cell cDNA using a forward primer (5'-CGGGATCCTTTTACTCCTAAACCACCTAA-3') and a reverse primer (5'-GGAATTCTCAGGGATGCTCAGAAGACGCAGA-3'), digested with BamHI and EcoRI, and integrated to the pGEX-6P-1 plasmid.

Quick freezing and freeze-fracture replica preparation

Quick freezing and freeze-fracture replica formation were carried out as described previously. In brief, samples were sandwiched and were frozen using an HPM 010 high-pressure freezing machine, and freeze-fracture replicas were prepared by electron-beam evaporation. Thawed freeze-fracture replicas were treated with 2.5% SDS in 0.1 M Tris-HCl (pH 7.4) at 60°C overnight.

Freeze-fracture replica labeling and quantitative analysis

After being treated with an SDS solution, the replicas were incubated with GST-tagged PH domain of TAPP-1(GST-TAPP-1) or non functional mutant probe (GST-TAPP-1(R211L); it was followed by rabbit Anti-GST antibody and colloidal gold conjugated protein A, and observed under transmission EM. The number of colloidal gold particles in EM images was counted manually, and areas were measured using Image J.

【Results】

GST-TAPP1-PH binds to PtdIns(3,4)P_2 in freeze-fracture replicas

By measuring the density of colloidal gold labels in freeze-fracture replicas of liposomes, we found that GST-TAPP1-PH binds to PtdIns(3,4)P_2 with the highest intensity (Figures 1A, B). In contrast, the mouse anti-PtdIns(3,4)P_2 antibody bound to phosphatidylinositol and several phosphoinositides other than PtdIns(3,4)P_2 with significant intensities. As the second line of evidence to show the binding specificity of GST-TAPP1-PH, we used a one amino acid-substituted mutant of TAPP1, R211L, that is deficient in binding to PtdIns(3,4)P_2. It was confirmed that GST-TAPP1-PH(R211L) does not bind to the freeze-fracture replica of PtdIns(3,4)P_2-containing liposomes (Figure 1C). The result indicated that GST-TAPP1-PH binds to PtdIns(3,4)P_2 in freeze-fracture replicas in the same manner as it does to PtdIns(3,4)P_2 in cells.

The mutant probe, GST-TAPP1-PH(R211L) is useful as a negative control to exclude non-specific binding in cellular samples, in which proteins as well as non-tested lipids might show affinity to GST-TAPP1-PH.

Treatment with H_2O_2 and PDGF increases PtdIns(3,4)P_2 in the plasma membrane

PtdIns(3,4)P_2 in culture cells was shown to increase significantly upon treatment with H_2O_2 and PDGF. We confirmed that H_2O_2 and PDGF increased PtdIns(3,4)P_2 in the plasma membrane of NIH3T3 cells by observing the distribution of expressed GFP-TAPP1-PH by fluorescence microscopy. In this cell type, the obvious distributional change of GFP-TAPP1-PH with H_2O_2 occurred only when it was used at 10 mM. Therefore, NIH3T3 cells treated with 10 mM H_2O_2 for 10 min and those treated with 5 ng/ml PDGF for 10 min were examined in subsequent experiments. Importantly, the mutant probe, GST-TAPP1-PH(R211L), did not show labeling in either. We also observed intracellular membranes of the stimulated cells by QF-FRL. In H_2O_2-treated cells, the inner and outer mitochondrial membranes were devoid of PtdIns(3,4)P_2 labeling (Figure 3A) whereas membranes in the Golgi occasionally showed a low level of labeling (Figure 3B). Additionally, some vesicular structures were labeled in the cytoplasmic leaflet (Figure 3C) but the identity of those structures could not be determined by morphological criteria.

【Discussion】

The present study showed that QF-FRL can label PtdIns(3,4)P_2 using recombinant GST-TAPP1-PH as the probe. In comparison to EM methods using ultrathin sections, an advantage of freeze-fracture EM is that membranes can be observed in two-dimensional planes. Because the method can visualize PtdIns(3,4)P_2 distribution in a high spatial resolution, it is expected to be useful to analyze phenomena occurring in small areas of membranes. Additionally, the QF-FRL method appears to label PtdIns(3,4)P_2 more efficiently than the method using sections: the increase of PtdIns(3,4)P_2 labeling after

stimulation with H_2O_2 and PDGF was approximately 45-fold and 18-fold, respectively, by QF-FRL, whereas by methods using sections, the increase (per unit length of the plasma membrane) was approximately four-fold and 2- to 2.5-fold when PtdIns(3,4)P_2 was directly labeled and approximately 10.4-fold and 5.2-fold when expressed GFP-TAPP1-PH was labeled. The result of QF-FRL agrees better with the drastic increase of PtdIns(3,4)P_2 that was measured biochemically. we believe that the method using TAPP1-PH for freeze-fracture replica labeling is an important tool that can be employed to analyze PtdIns(3,4)P_2 in various cellular context.