

主論文の要旨

**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 3,4-二リン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)P₂ being revealed, the need to determine where and how PtdIns(3,4)P₂ is located in the cell is more urgent than ever. Although methods to quantify the amount of PtdIns(3,4)P₂ in isolated samples have been developed, considering the quick time course over which PtdIns(3,4)P₂ is thought to be generated and hydrolyzed, microscopic methods to observe its distribution are essential for understanding PtdIns(3,4)P₂ 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)P₂ 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)P₂ 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)P₂ with a higher specificity than a commercially available anti-PtdIns(3,4)P₂ 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)P₂ 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 H₂O₂ 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)P₂ 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)P₂ with the highest intensity (Figures 1A, B). In contrast, the mouse anti-PtdIns(3,4)P₂ antibody bound to phosphatidylinositol and several phosphoinositides other than PtdIns(3,4)P₂ 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)P₂. It was confirmed that GST-TAPP1-PH(R211L) does not bind to the freeze-fracture replica of PtdIns(3,4)P₂-containing liposomes (Figure 1C). The result indicated that GST-TAPP1-PH binds to PtdIns(3,4)P₂ in freeze-fracture replicas in the same manner as it does to PtdIns(3,4)P₂ 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 H₂O₂ and PDGF increases PtdIns(3,4)P₂ in the plasma membrane

PtdIns(3,4)P₂ in culture cells was shown to increase significantly upon treatment with H₂O₂ and PDGF. We confirmed that H₂O₂ and PDGF increased PtdIns(3,4)P₂ 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 H₂O₂ occurred only when it was used at 10 mM. Therefore, NIH3T3 cells treated with 10 mM H₂O₂ 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 H₂O₂-treated cells, the inner and outer mitochondrial membranes were devoid of PtdIns(3,4)P₂ 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)P₂ 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)P₂ 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)P₂ more efficiently than the method using sections: the increase of PtdIns(3,4)P₂ labeling after

stimulation with H₂O₂ 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)P₂ 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)P₂ 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)P₂ in various cellular context.