

Phosphatidylinositol 4-Phosphate 5-Kinase α Is a Downstream Effector of the Small G Protein ARF6 in Membrane Ruffle Formation

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Summary

Synthesis of phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂], a signaling phospholipid, is primarily carried out by phosphatidylinositol 4-phosphate 5-kinase [PI(4)P5K], which has been reported to be regulated by RhoA and Rac1. Unexpectedly, we find that the GTP γ S-dependent activator of PI(4)P5K α is the small G protein ADP-ribosylation factor (ARF) and that the activation strictly requires phosphatidic acid, the product of phospholipase D (PLD). In vivo, ARF6, but not ARF1 or ARF5, spatially coincides with PI(4)P5K α . This colocalization occurs in ruffling membranes formed upon AIF₄ and EGF stimulation and is blocked by dominant-negative ARF6. PLD2 similarly translocates to the ruffles, as does the PH domain of phospholipase C δ 1, indicating locally elevated PI(4,5)P₂. Thus, PI(4)P5K α is a downstream effector of ARF6 and when ARF6 is activated by agonist stimulation, it triggers recruitment of a diverse but interactive set of signaling molecules into sites of active cytoskeletal and membrane rearrangement.

Introduction

Phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂] plays crucial roles in various cellular signaling pathways. One of the best understood of its functions is to serve as a precursor of two well-characterized second messengers produced by phospholipase C (Berridge, 1987): diacylglycerol, which activates protein kinase C (Nishizuka,

1984), and inositol 1,4,5-trisphosphate, which mobilizes intracellular Ca²⁺ (Berridge and Irvine, 1984). PI(4,5)P₂ is also metabolized by phosphoinositide 3-kinases to phosphatidylinositol 3,4,5-trisphosphate (Auger et al., 1989; reviewed by Cantley et al., 1991), which plays pivotal roles in mitogenic signaling, cytoskeletal remodeling, and vesicular trafficking (reviewed by Wymann and Pirola, 1998). Moreover, PI(4,5)P₂ directly regulates several proteins and enzymes that are involved in diverse cellular processes: it binds actin-binding proteins and thereby regulates actin cytoskeleton reorganization (Lassing and Lindberg, 1985; Janmey and Stossel, 1987; Fukami et al., 1992); it is required as a cofactor for the activation of phospholipase D (PLD) (Brown et al., 1993; Hammond et al., 1995; Colley et al., 1997; Hammond et al., 1997); and finally, it stimulates both guanine nucleotide exchange on ARF (Terui et al., 1994; Randazzo, 1997) and the ARF GTPase-activating protein (Randazzo and Kahn, 1994). Thus, PI(4,5)P₂ is a remarkably versatile phospholipid.

In mammalian cells, PI(4,5)P₂ is synthesized through phosphorylation of phosphatidylinositol 4-phosphate [PI(4)P] by PI(4)P 5-kinases [PI(4)P5Ks] at the D-5 position of the inositol ring (reviewed by Toker, 1998). Type I and type II of PI(4)P5Ks were initially defined (Loijens et al., 1996) and consisted of three isoforms of type I, α , β , and γ (Ishihara et al., 1996, 1998; Loijens and Anderson, 1996), and three isoforms of type II (Boronnikov and Anderson, 1995; Divecha et al., 1995; Castellino et al., 1997; Itoh et al., 1998). Reinvestigation of the substrate specificity of PI(4)P5Ks by Rameh et al. (1997), however, revealed that the type II PI(4)P5Ks catalyze phosphorylation of a novel phosphoinositide phosphatidylinositol 5-phosphate at the D-4 position of the inositol ring, leading to the revised conclusion that the type II enzymes are actually phosphatidylinositol 5-phosphate 4-kinases. Thus, the PI(4)P5K family now comprises just the three isoforms, α , β , and γ .

PI(4)P5K has been reported to be activated in vitro by acid phospholipids—specifically phosphatidic acid (PA) (Moritz et al., 1992; Jenkins et al., 1994; Ishihara et al., 1996, 1998) and phosphatidylserine (PS) (Cochet and Chambaz, 1986). PI(4)P5K was also anticipated to be regulated by a GTP-binding protein, based on the finding that GTP γ S stimulates PI(4,5)P₂ production in human placenta, rat brain, and rat liver membranes (Urumow and Wieland, 1986; Smith and Chang, 1989; Urumow and Wieland, 1990). Chong et al. (1994) and Hartwig et al. (1995) reported that the small G proteins RhoA and Rac1 respectively stimulate PI(4,5)P₂ production in lysates from mouse fibroblasts and in permeabilized platelets, which suggested that they are direct activators of PI(4)P5K. However, such direct activation has never actually been formally demonstrated.

In this study we have purified the GTP γ S-dependent activator of PI(4)P5K α from bovine brain cytosol and unexpectedly identified it as the small G protein ADP-ribosylation factor (ARF). Investigation of the interaction in vivo provides evidence that PI(4)P5K α is a physiological downstream effector specifically of ARF6 and that

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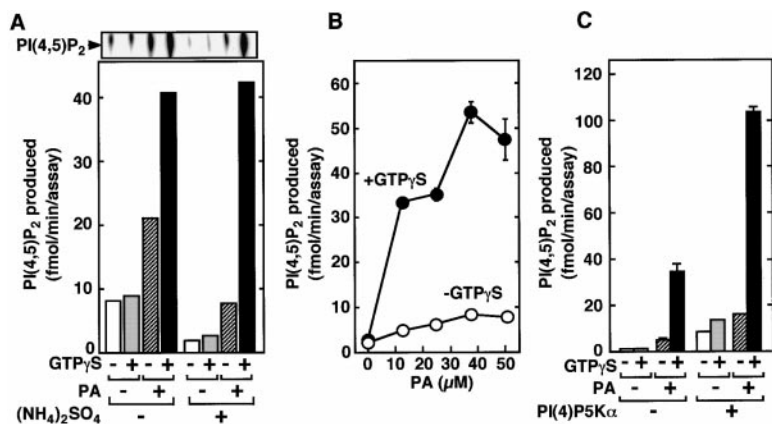


Figure 1. Effects of GTP γ S and PA on PI(4,5)P₂ Production in Bovine Brain Cytosol (A) Bovine brain cytosol was incubated alone (open columns), with 40 μ M of GTP γ S (stippled columns), with 50 μ M PA (hatched columns), or with both (solid columns), in the absence (left four columns) or presence (right four columns) of 0.2 M ammonium sulfate, following which the amounts of [³²P]PI(4,5)P₂ generated were determined as described in Experimental Procedures. The upper panel shows an autoradiogram of the [³²P]PI(4,5)P₂ produced, which was separated by TLC. (B) Bovine brain cytosol was incubated alone (open circles) or with 40 μ M of GTP γ S (closed circles) in the presence of the indicated concentrations of PA and of 0.2 M ammonium sulfate as above.

(C) Vehicle (left four columns) or the COS-7 cell-expressed recombinant mouse PI(4)P5K α (right four columns) was added to bovine brain cytosol, which was then assayed in the presence of 0.2 M ammonium sulfate as in (A). The results shown in (A), and (B) and (C) are from a single experiment in single and duplicate, respectively, representative of five performed. The error bars represent the differences of duplicate determinations.

through its product PI(4,5)P₂, it is likely to play a role in membrane ruffle formation as induced by epidermal growth factor (EGF).

Results

GTP γ S and PA Synergistically Increase PI(4,5)P₂ Production in Bovine Brain Cytosol

We began our studies by reexamining the requirements for PI(4)P5K activation, since the stimulation of PI(4,5)P₂ production by PA and PS had been assessed using cytosol or recombinant PI(4)P5Ks (Cochet and Chambaz, 1986; Moritz et al., 1992; Jenkins et al., 1994; Ishihara et al., 1996, 1998), while the stimulation by GTP γ S had been demonstrated using membrane preparations (Urumow and Wieland, 1986; Smith and Chang, 1989; Urumow and Wieland, 1990). Unexpectedly, although addition of PA to bovine brain cytosol increased PI(4,5)P₂ production by 2- to 3-fold, significant stimulation by GTP γ S alone was not observed (Figure 1A). However, GTP γ S did strongly increase PI(4,5)P₂ production when PA was present. In the presence of 0.2 M ammonium sulfate, similar effects of GTP γ S and/or PA were observed, but the basal level was lower and therefore the relative stimulation much greater. This appeared to be due to inhibition of enzymes that were further metabolizing PI(4,5)P₂ produced (data not shown). Accordingly, ammonium sulfate was thereafter included in the crude assay system. The extent of increase in PI(4,5)P₂ production by GTP γ S was critically dependent upon the presence of PA (Figure 1B). Thus, GTP γ S absolutely required the presence of PA to stimulate PI(4,5)P₂ production in bovine brain cytosol, and in combination the two stimulators were synergistic.

The increase in PI(4,5)P₂ production by GTP γ S and PA appeared to be, at least in part, attributable to the activation of PI(4)P5K α , inasmuch as a similar but augmented response was observed when recombinant mouse PI(4)P5K α was added to the cytosol (Figure 1C). These results, taken together, demonstrated that a

GTP γ S-dependent activator of PI(4)P5K α that functions only in the presence of PA exists in bovine brain cytosol.

Purification and Identification of a GTP γ S-Dependent PI(4)P5K α Activator

By assaying recombinant mouse PI(4)P5K α in combination with bovine brain cytosol sequentially fractionated by column chromatographies, a GTP γ S-dependent activator of PI(4)P5K α was purified (Figures 2A–2E). On the final Superdex 75 HR 10/30 column, the activator eluted in a single peak as a 21 kDa protein (Figure 2E) and SDS-PAGE analysis (Figure 2F) revealed that it had been purified to near homogeneity. The purification was about 400-fold with 2.4% yield (data not shown), implying that the activator is abundant in bovine brain cytosol.

To identify the activator, amino acid sequences of trypsin-digested peptide fragments of the purified protein were analyzed. The sequences of the six peptide fragments analyzed exhibited identity to the small G proteins ARF1 and ARF3, which are identical over the regions covered by the peptide sequences (Figure 3A). The 21 kDa protein eluted from the Superdex 75 HR 10/30 column was strongly recognized by an anti-ARF antibody in parallel with the PI(4)P5K α activation (Figure 3B). In contrast, anti-RhoA, anti-Rac1, and anti-Cdc42 antibodies failed to react with the protein (Figure 3C). These results demonstrated that ARF is the most abundant GTP γ S-dependent activator of PI(4)P5K α in brain cytosol.

Reconstitution of Synergistic Activation of PI(4)P5K α by PA and ARF, but Not by RhoA, Rac1, and Other Phospholipids

When bacterial recombinant PI(4)P5K α and myristoylated ARF1 (myr-ARF1) were combined in the presence of PA (Figure 4A), myr-ARF1 activated the enzyme in the presence of GTP γ S, but not GDP, and in a concentration-dependent manner, although the concentrations of myr-ARF1 required were higher than those for the native protein. Nonetheless, the results provide evidence that

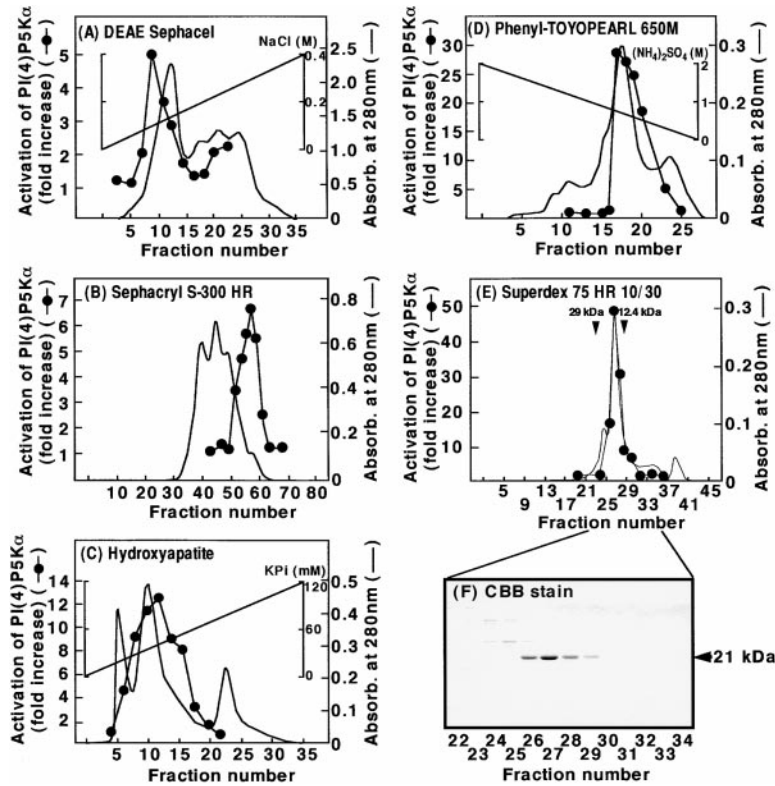


Figure 2. Purification of a GTP- γ S-Dependent Activator of PI(4)P5K α from Bovine Brain Cytosol

Bovine brain cytosol proteins were precipitated with ammonium sulfate at 25%–60% saturation and subjected to sequential chromatography on DEAE Sephacel (A), Sephacryl S-300 HR (B), Hydroxyapatite (C), Phenyl-TOYOPEARL 650M (D), and Superdex 75 HR 10/30 columns (E). Each column fraction was assessed for activation of COS-7 cell-expressed recombinant mouse PI(4)P5K α in the presence of 0.2 M ammonium sulfate, 40 μ M GTP- γ S, and 50 μ M PA as in Figure 1. Fractions eluted from the Superdex 75 HR 10/30 column were additionally analyzed by SDS-PAGE/CBB staining (F). The results shown are from a single experiment representative of three performed with independent preparations of bovine brain cytosol.

the activation by ARF1 of PI(4)P5K α is attributable to their direct interaction. ARF5 and ARF6, class II and III ARFs, respectively, also activated PI(4)P5K α (Figure 4B).

The identification of ARF as the primary endogenous activator of PI(4)P5K was surprising because RhoA and Rac1 had been previously implicated in this role (Chong et al., 1994; Hartwig et al., 1995). To investigate whether PI(4)P5K α is also activated by these small G proteins, the ability of recombinant RhoA and Rac1 purified from Sf9 cells to stimulate bacterial recombinant PI(4)P5K α was compared with that of ARF1 (Figure 4C). Whereas ARF1 activated PI(4)P5K α , RhoA and Rac1 were without effect. Similar results were obtained using mammalian recombinant PI(4)P5K α (data not shown). The recombinant RhoA and Rac1 were clearly active, since they stimulated human PLD1a in the presence of GTP- γ S (data not shown). These results, taken together, provide evidence that ARF is the specific activator of PI(4)P5K α in vitro.

Since it has been reported that PS, as well as PA, activates PI(4)P5K (Cochet and Chambaz, 1986), phospholipid specificity in the activation of PI(4)P5K α was assessed (Figure 4D). Phosphatidylethanolamine (PE), phosphatidylcholine (PC), PI, and lyso-PA (LPA) did not significantly activate PI(4)P5K α , which was assayed in combination with myr-ARF1, irrespective of the presence of GDP or GTP- γ S. Although PS, like PA, modestly activated the enzyme in the presence of GDP, it failed to synergize with GTP- γ S-bound ARF1. Thus, synergistic activation with ARF1 was specific to PA. This PA effect seemed to be attributable to direct action on PI(4)P5K α

instead of ARF since PA did not affect [³⁵S]GTP- γ S binding to ARF1 (Figure 4E) and PI(4)P5K α activity was preferentially precipitated by phospholipid vesicles containing PA (Figure 4F).

ARF6-Dependent Translocation of PI(4)P5K α to Ruffling Membranes with Concomitant PI(4,5)P₂ Formation and Cotranslocation of PLD2

To address which isoform of ARF is the physiological activator of PI(4)P5K α , subcellular localization of C-terminally hemagglutinin (HA) epitope-tagged ARFs (ARFs-HA) and N-terminally myc epitope-tagged PI(4)P5K α [myc-PI(4)P5K α] coexpressed in HeLa cells were visualized using indirect immunofluorescence microscopy, under the assumption that activation will take place only if colocalization occurs (Figure 5A). In unstimulated cells, ARF1 was localized exclusively to perinuclear Golgi membranes, ARF5 to the nucleus and perinuclear membranes and vesicles, and ARF6 throughout the cytoplasm and at the plasma membrane, as reported previously (Hosaka et al., 1996; Radhakrishna et al., 1996). PI(4)P5K α localized to the plasma membrane and also to punctate structures dispersed throughout the cytoplasm. Upon stimulation by AIF₄, cells overexpressing ARF6 formed membrane ruffles and ARF6 became concentrated in the ruffles, as previously reported (Radhakrishna et al., 1996, 1999). Strikingly, PI(4)P5K α cotranslocated with ARF6 to the ruffles. Neither colocalization, membrane ruffles, nor obvious translocation of PI(4)P5K α

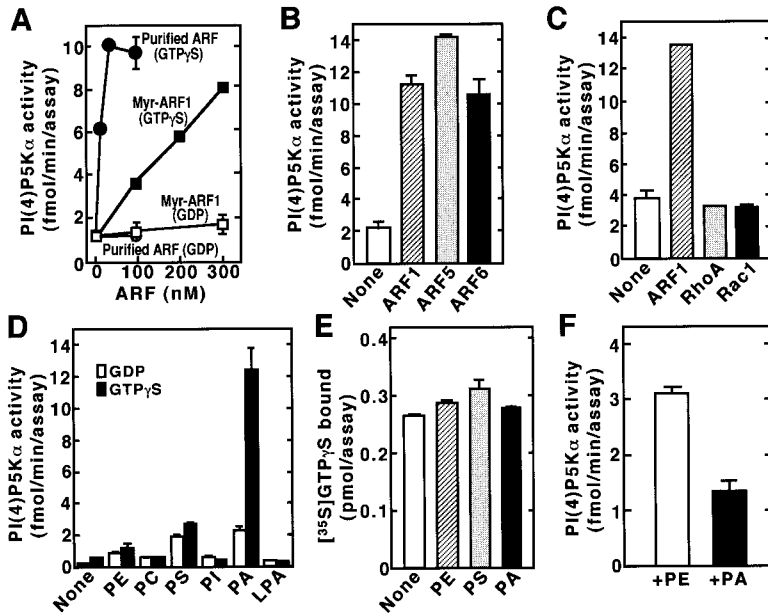


Figure 4. Reconstitution of Synergistic Activation of PI(4)P5K α by ARFs and PA, but Not by RhoA, Rac1, and Other Phospholipids

(A) Recombinant PI(4)P5K α (15 nM) and the indicated concentrations of myr-ARF1 (squares), both of which were purified from *E. coli*, or purified ARF from bovine brain cytosol (circles) were combined and assayed for 25 min at 37°C with 40 μ M of GDP (open symbols) or GTP γ S (closed symbols) in the presence of 50 μ M PA following which [³²P]PI(4,5)P₂ production was quantitated.

(B) Bacterial recombinant PI(4)P5K α (15 nM) was assayed in combination with vehicle or 30 nM of recombinant ARF1, ARF5, or ARF6 purified from COS-7 cells in the presence of 40 μ M of GTP γ S and 50 μ M PA.

(C) Bacterial recombinant PI(4)P5K α (15 nM) was assayed in combination with vehicle or 500 nM of recombinant ARF1, RhoA, or Rac1 purified from Sf9 cells in the presence of 40 μ M of GTP γ S and 50 μ M PA.

(D) Bacterial recombinant PI(4)P5K α (15 nM) was combined with myr-ARF1 (300 nM) and assayed in the presence of 12.5 μ M of the indicated phospholipids and 40 μ M of GDP

(open columns) or GTP γ S (solid columns). In this experiment, the PI(4)P substrate was used at 12.5 μ M. (E) Bacterial myr-ARF1 (3 μ M) was incubated for 25 min at 37°C with 10 μ M of [³⁵S]GTP γ S in the presence of 12.5 μ M of the indicated phospholipids. [³⁵S]GTP γ S bound to myr-ARF1 was determined as described in Experimental Procedures.

(F) Bacterial recombinant PI(4)P5K α (15 nM) was incubated for 20 min at room temperature with 150 μ M PE/20 μ M PC vesicles containing 200 μ M of PE (open column) or PA (solid column) in the presence of 100 mM NaCl and 5 mg/ml BSA. After centrifugation, PI(4)P5K activity in the supernatant was determined as described in Experimental Procedures.

Shown are the results from a single experiment in duplicate and representative of five with similar results. The error bars represent the differences of duplicate determinations.

previous report (Colley et al., 1997). With EGF stimulation, the overexpressed PLD2 translocated to the ruffling membranes as visualized at 7 min and colocalized with PI(4)P5K α . As before, ruffle formation was transient, and both proteins became redistributed in parallel with concomitant retraction of the ruffles. In contrast, PLD1 did not alter its perinuclear distribution upon EGF stimulation (data not shown). These results demonstrate that a mechanism to locally generate PA is established during membrane ruffle formation and suggests that it plays a role in the activation of PI(4)P5K α .

Requirement of Local Production of PI(4,5)P₂ for Membrane Ruffling Formation

Growth factor-induced membrane ruffling formation is also regulated by the Rho family GTPase Rac1 (Ridley et al., 1992). Radhakrishna et al. (1999) have recently reported that the dominant-negative ARF6 mutant inhibits the membrane ruffling formation induced by the constitutively active Rac1 mutant. Consistent with these reports, membrane ruffles were formed by the active Rac1 mutant, Rac1 G12V, which colocalized to the ruffles with coexpressed ARF6 and PI(4)P5K α , and the dominant-negative ARF6 mutant, ARF6 N122I, inhibited both the membrane ruffling formation and the translocation of PI(4)P5K α induced by Rac1 G12V (Figure 6A), as was observed in EGF-stimulated cells (Figure 5C). Furthermore, microinjection of an anti-PI(4,5)P₂ antibody, AM212 (Miyazawa et al., 1988), almost completely inhibited the membrane ruffling formation induced by Rac1 G12V (Figure 6B). These observations suggest that

the EGF-induced signaling pathway that induces ARF6-dependent PI(4)P5K α translocation to the plasma membrane is mediated by Rac1 and that the local production of PI(4,5)P₂ at the plasma membrane is, at least in part, involved in the membrane ruffling formation. Although consistent with a previous report (Ridley et al., 1992), the dominant-negative Rac1 mutant, Rac1 T17N, inhibited EGF-induced membrane ruffling, it nonetheless failed to interfere with the EGF-induced cotranslocation of ARF6 and PI(4)P5K α to the plasma membrane and concomitant PI(4,5)P₂ production, the latter of which was detected using the anti-PI(4,5)P₂ antibody (Figure 6C). In mock transfected cells, both the membrane ruffling and cotranslocation of ARF6 and PI(4)P5K α to the ruffles were observed (data not shown). These results, taken together, lead to the conclusion that (1) the local generation of PI(4,5)P₂ by activated PI(4)P5K α at the plasma membrane is essential, but not sufficient, for membrane ruffling formation; (2) ARF6-dependent translocation of PI(4)P5K α to the plasma membrane involves Rac1-dependent and -independent mechanisms; and (3) in addition to the ARF6-dependent local production of PI(4,5)P₂, another Rac1-dependent, ARF6-independent pathway is required for the membrane ruffling formation induced by EGF stimulation, as illustrated in Figure 7.

Discussion

In the present study, we demonstrate that the small G protein ARF directly activates PI(4)P5K α in concert with PA. Furthermore, we provide evidence that PI(4)P5K α

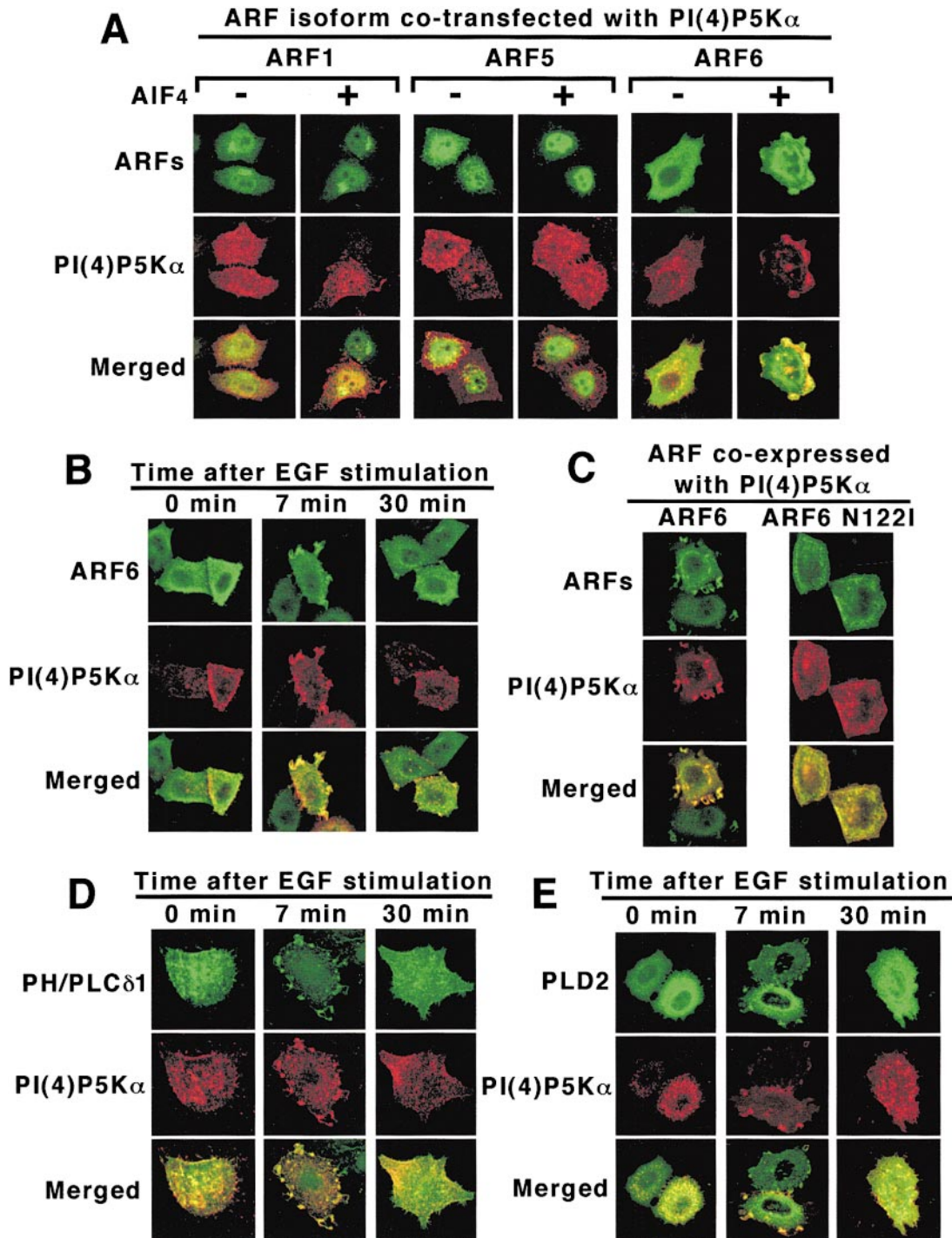


Figure 5. ARF6-Dependent Translocation of PI(4)P5K α to Ruffling Membranes with Concomitant PI(4,5)P₂ Formation and Cotranslocation of PLD2 (A) Myc-PI(4)P5K α was cotransfected with ARF1-HA, ARF5-HA, or ARF6-HA in HeLa cells. After 6 hr of additional culture in the presence of 5% FCS, cells were treated without or with AIF₄ (30 mM sodium fluoride plus 50 μ M aluminum chloride) at 37°C for 30 min, and localization of ARFs and PI(4)P5K α was then visualized as described under Experimental Procedures.

(B) HeLa cells cotransfected with myc-PI(4)P5K α and ARF6-HA were cultured in DMEM without FCS for 36 hr, stimulated with 10 ng/ml of EGF at 37°C for the times indicated, then processed as in (A).

(C) Wild-type ARF6-HA or the dominant-negative mutant ARF6 N122I-HA was cotransfected with myc-PI(4)P5K α in HeLa cells. Cells were further cultured as in (B), then stimulated with 10 ng/ml of EGF at 37°C for 7 min and imaged as in (A).

(D) HeLa cells cotransfected with GFP-PH/PLC δ 1, myc-PI(4)P5K α , and ARF6, were cultured and stimulated with EGF as in (B), and imaged as in (A).

(E) HeLa cells cotransfected with HA-PLD2, myc-PI(4)P5K α , and ARF6, were cultured and stimulated with EGF as in (B), and imaged as in (A).

Shown is one experiment representative of five performed.

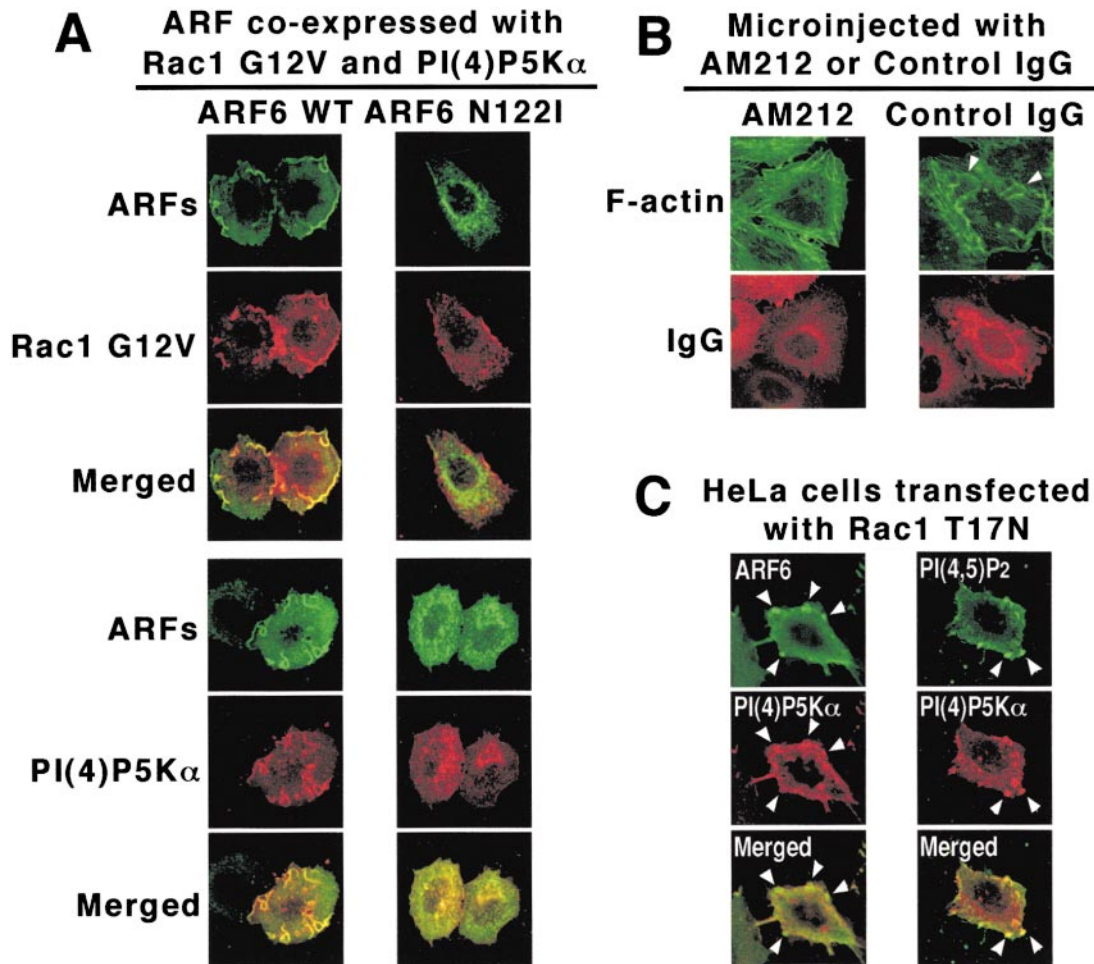


Figure 6. Analysis for the Mechanisms by which Membrane Ruffles Are Formed

(A) Myc-Rac1 G12V was cotransfected with myc-PI(4)P5K α and with wild type ARF6-HA or the dominant-negative mutant ARF6 N122I-HA in HeLa cells. After culture in DMEM without FCS for 36 hr, localization of the tagged proteins was visualized as described under Experimental Procedures.

(B) HeLa cells were transfected with Rac1 G12V, cultured 34 hr, then microinjected with 2 mg/ml of control mouse IgG or the monoclonal anti-PI(4,5)P₂ antibody (AM212). After 2 hr, actin filaments and IgG were visualized as described under Experimental Procedures.

(C) HeLa cells were transfected with Rac1 T17N (shown) or mock (not shown), cultured for 32 hr, and then microinjected with expression vectors for ARF6 and PI(4)P5K α . After 4 hr, cells were stimulated with 10 ng/ml of EGF at 37°C for 7 min, and localization of ARF6, PI(4)P5K α and PI(4,5)P₂ were visualized as described under Experimental Procedures.

Shown is one experiment representative of five performed.

is a downstream effector of ARF6, and that it is synergistically activated by ARF6 and the PLD2 product PA to increase local production of PI(4,5)P₂ in the plasma membrane upon EGF stimulation, which in turn promotes membrane ruffling formation in concert with the Rac1-specific pathway.

Activation Mechanism of PI(4)P5K by PA and Small G Proteins

In the present study, we identified ARF as the direct activator of PI(4)P5K α in vitro (Figures 3 and 4). As reported previously for the activation of PLD (Brown et al., 1993; Cockcroft et al., 1994) and for promoting vesicular transport (Balch et al., 1992; Kahn et al., 1992; Lenhard et al., 1992), myristoylation of ARF also appears to be required for the activation of PI(4)P5K α , since the recombinant myr-ARF1, which is partially (10%–50%) myristoylated in *E. coli* coexpressing N-myristoyltransferase

(Liang and Kornfeld, 1997), was considerably less effective than the native proteins (Figure 4A). Since the function of myristoylation is to promote membrane localization of GTP-bound ARF, this suggests, in combination with the absolute requirement of the membrane lipid PA, that activation of PI(4)P5K α takes place only at membrane surfaces where activated ARF and PLD2 (as the source for PA) coincide.

RhoA and Rac1 had been reported to activate PI(4)P5K in mouse fibroblast lysates and permeabilized platelets, respectively (Chong et al., 1994; Hartwig et al., 1995). Under the conditions employed in this study, however, they failed to activate PI(4)P5K α (Figure 4C). These results indicate that the stimulation by RhoA and Rac1 of PI(4)P5K reported must reflect an indirect mechanism. Alternatively, RhoA and Rac1 may regulate an unidentified isoform of PI(4)P5K, which is supported by a previous report that RhoA physically associates with a

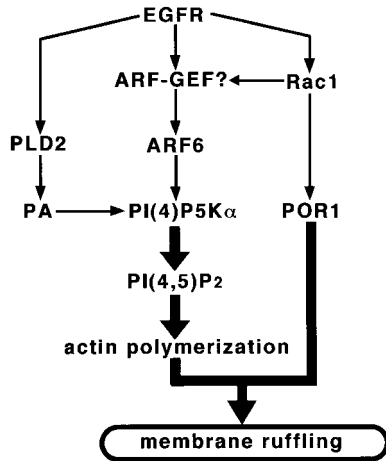


Figure 7. Working Hypothesis for the Mechanisms of PI(4)P5K α Activation and of Membrane Ruffling Formation upon EGF Stimulation See text for details.

PI(4)P5K that is insensitive to PA (Ren et al., 1996). It is also conceivable that their target is the β or γ isoform of PI(4)P5K, although unpublished data from our lab suggests that this is not the case. The nature of the PI(4)P5K that Rac1 associates with in rat liver cytosol and Rat1a fibroblast lysates in a GTP-independent manner (Tolias et al., 1995, 1998) remains undetermined.

It has been reported that GTP γ S significantly activates PI(4)P5K in membranes (Urumow and Wieland, 1986; Smith and Chang, 1989; Urumow and Wieland, 1990). We found the stimulation of PI(4,5)P₂ formation by GTP γ S alone to be minimal, if any, in the cytosol (Figure 1). Since the addition of PA to the cytosol allowed GTP γ S to stimulate PI(4,5)P₂ formation (Figure 1) and GTP γ S-bound ARF activated PI(4)P5K α in the presence, but not in the absence, of PA in the reconstituted system (Figure 4D), the lack of the ability of GTP γ S to activate PI(4)P5K in the cytosol is clearly attributable to the absence of PA; in contrast, membranes constitutively contain basal levels of PA, and therefore addition of GTP γ S alone to them increases PI(4,5)P₂ production.

The requirement of phospholipids for ARF activation of PI(4)P5K α was specific to PA (Figure 4D). This specific effect of PA cannot be simply explained by a physical effect on the substrate vesicle structure through its negative charge, since the other negatively charged phospholipids tested, PS, PI, and LPA, were without effect. It is also unlikely that PA has a direct effect on ARF, since PA did not affect [³⁵S]GTP γ S binding to ARF1 under these conditions (Figure 4E) nor is PA required for another ARF downstream effector, PLD (Hammond et al., 1997). The most likely explanation is that PA directly stimulates PI(4)P5K α : PI(4)P5K α binds to phospholipid vesicles containing PA, but not to vesicles lacking PA (Figure 4F). This notion is also supported by the fact that even by itself PA stimulates PI(4)P5K α , but it does not stimulate PI(5)P4K α (data not shown). These observations lead us to speculate that binding of PA to PI(4)P5K α causes a conformational change in the enzyme, which in turn allows it to interact with the active form of ARF, thereby leading to synergistic stimulation.

Relationship between Rac1 and ARF6 in the Membrane Ruffling Signaling Pathway

Franco et al. (1999) recently identified the guanine nucleotide exchange factor for ARF6 (EFA6) and found that expression of EFA6 induces membrane ruffles. Furthermore, they have demonstrated that both dominant-negative mutants of ARF6 and Rac1 inhibited the EFA6-induced membrane ruffles. From these results, they hypothesized that EFA6 activates both ARF6 and Rac1 in parallel pathways. This hypothesis (Rac1-independent activation of ARF6) is consistent with the results showing the failure of Rac1 T17N to inhibit the ARF6-dependent signaling leading to the activation of PI(4)P5K α (Figure 6C). In addition, ARF6 could also be activated by a Rac1-dependent mechanism, which may involve an ARF-GEF (Figure 7), based on the result that ARF6 N122I inhibited the Rac1 G12V-induced translocation of PI(4)P5K α to the plasma membrane (Figure 6A). Furthermore, it could be speculated that membrane ruffling formation induced by EGF requires two parallel pathways; one being the ARF6-dependent activation of PI(4)P5K α and the consequent PI(4,5)P₂ elevation, and the other a Rac1-specific, ARF6-independent pathway possibly mediated by POR1, a Rac1-interacting protein involved in membrane ruffling (D'Souza-Schorey et al., 1997) (Figure 7). This hypothesis is supported by the findings that the dominant-negative ARF6 N122I and the anti-PI(4,5)P₂ antibody inhibited the Rac1 G12V-induced membrane ruffling formation (Figures 6A and 6B), while EGF stimulation still caused the cotranslocation of PI(4)P5K α and ARF6 to the plasma membrane where PI(4,5)P₂ was produced, without induction of the membrane ruffling in cells overexpressing the dominant-negative Rac1 T17N (Figure 6C). The observation by D'souza-Schorey et al. (1997) that ARF6 and POR1 synergistically induce membrane ruffling also supports this model.

Relationship of PLD2 to the Activation of PI(4)P5K α

Both PLD1 and PLD2 are ARF responsive (Hammond et al., 1995, 1997; Sung et al., 1999a, 1999b), although the details of this regulation in vivo are not well understood. In this study, PLD2 was found to cotranslocate with PI(4)P5K α to the ruffling membranes formed upon EGF stimulation (Figure 5E), whereas PLD1 did not alter its perinuclear location, suggesting that PLD2, but not PLD1, is involved in PI(4)P5K α activation in this setting. This conclusion is also supported by the observation that EGF stimulation of cells overexpressing PLD2 stimulates PLD activity (Slaaby et al., 1998), and that overexpression of PLD2 in many cell types results in actin cytoskeletal rearrangements (Colley et al., 1997).

It should be noted, though, that PLD1 may activate another PI(4)P5K isoform(s) different from PI(4)P5K α , or even PI(4)P5K α itself in a different setting, since PI(4)P5K β has been reported in association with receptor signaling and endocytosis (Davis et al., 1997), and one or more of the PI(4)P5K isoforms is involved in the priming of exocytosis (Hay et al., 1995), either of which may involve PLD1. Nevertheless, although further investigation is required to explore the involvement of PLD2 in PI(4)P5K α activation, this report demonstrates a novel physiological function for PLD2 and it sheds insight into the role of PLD2 in EGF-stimulated cellular signaling.

Novel Physiological Function of PI(4)P5K α

It is firmly established that regulated exocytosis, such as ATP-dependent, Ca²⁺-activated neurotransmitter secretion in PC12 cells (Hay et al., 1995) and enzyme release in HL-60 cells (Fensome et al., 1996), appears to be mediated by PI(4,5)P₂ produced by the action of PI(4)P5K. In addition to this role, we provide evidence in the present study for a novel function for PI(4)P5K α , i.e., involvement in membrane ruffle formation as induced by EGF stimulation, which also appears likely to be mediated by its product PI(4,5)P₂, inasmuch as the local production of PI(4,5)P₂ at the ruffling membrane and the inhibition by the anti-PI(4,5)P₂ antibody of the Rac1 G12V-induced membrane ruffling formation were observed (Figures 5D and 6B). This idea is consistent with many reports linking PI(4,5)P₂ to reorganization of the actin cytoskeleton through the regulation of actin-binding proteins (Lassing and Lindberg, 1985; Janmey and Stossel, 1987; Fukami et al., 1992). Most noteworthy of these, the PI(4,5)P₂-regulated actin severing and capping protein, gelsolin, has been found by Radhakrishna et al. (1996) to colocalize with ARF6 at the ruffles formed upon AIF₄ stimulation and by Steed et al. (1996) to associate with PLD2. Moreover, Azuma et al. (1998) reported that gelsolin-null dermal fibroblasts have a markedly reduced ruffling response to EGF stimulation. Although the detailed mechanism by which gelsolin mediates membrane ruffling formation remains to be clarified, it is presently the best candidate as a target protein for PI(4,5)P₂ in this setting.

Experimental Procedures

Reagents and Antibodies

Phospholipids, except for PI(4)P and LPA, were purchased from Avanti Polar Lipids. PI(4)P, LPA, ATP, and GTP γ S were obtained from Sigma, [γ -³²P]ATP and [³⁵S]GTP γ S from Amersham Life Technologies, and EGF from Takara Shuzo Co., Ltd (Shiga, Japan). Polyclonal rabbit anti-RhoA, anti-Rac1, and anti-Cdc42 antibodies, and a monoclonal mouse anti-myc antibody (9E10) were obtained from Santa Cruz Biotechnology. Monoclonal rat anti-HA and mouse anti-FLAG M2 antibodies were from Boehringer Mannheim and Eastman Kodak Co., respectively. Cy3- and FITC-conjugated secondary antibodies and Alexa 488 phalloidin were from Jackson ImmunoResearch Laboratories and Molecular Probes, respectively. A rabbit anti-ARF antiserum was raised against a synthetic peptide, corresponding to amino acid residues 23–36 of ARF1–5 and 19–32 of ARF6. A monoclonal mouse anti-PI(4,5)P₂ antibody (AM212) was a generous gift of Dr. Umeda, The Tokyo Metropolitan Institute of Medical Science, Japan (Miyazawa et al., 1988).

Plasmid Construction

PI(4)P5K α cDNA was amplified from mouse brain total RNA using RT-PCR and the cDNA sequence (Ishihara et al., 1996) verified. The mouse ARF cDNAs were previously described (Hosaka et al., 1996). An N122I mutation was introduced into ARF6 using a PCR-based strategy (Toda et al., 1999). pTB701-FLAG and pcDNA3-FLAG were used to construct mammalian expression vectors for N- and C-terminally FLAG epitope-tagged PI(4)P5K α [FLAG-PI(4)P5K α] and ARFs (ARFs-FLAG), respectively. pcDNA3-FLAG was constructed by insertion of the FLAG epitope sequence into pcDNA3 (Invitrogen). pFLAG-MAC (Eastman Kodak Co.) and pET20b (Novagen) were used to construct bacterial expression vectors for FLAG-PI(4)P5K α and C-terminally 6-His-tagged ARF1 (ARF1-His), respectively. The bacterial expression vector for N-myristoyltransferase, pBB131, was a generous gift of Dr. Gordon, Washington University (Duronio et al., 1990).

Myc-tagged mammalian expression vectors for PI(4)P5K α and

Rac1 G12V were generated using pcDNA3-myc, which was constructed by insertion of a myc epitope-encoding linker into pcDNA3. An HA-tagged version of PI(4)P5K α was generated using pTB701-HA. The construction of plasmids for ARFs-HA, ARF6 N122I-HA, and HA-PLD2 were previously described (Hosaka et al., 1996; Colley et al., 1997; Toda et al., 1999). The expression vectors for GFP-PH/PLC δ 1 and FLAG-Rac1 T17N were generous gifts of Dr. Yagisawa, Himeji Institute of Technology, Japan (Fujii et al., 1999) and of Dr. Kaziro, Tokyo Institute of Technology, Japan respectively.

Preparation of Recombinant PI(4)P5K α , ARFs, RhoA, and Rac1

To prepare mammalian recombinant FLAG-PI(4)P5K α and ARFs-FLAG, pTB701-FLAG-PI(4)P5K α and pcDNA3-ARFs-FLAG were transfected into COS-7 cells by electroporation and the proteins purified as previously described (Yamazaki et al., 1999).

FLAG-PI(4)P5K α was also inducibly expressed in *E. coli* with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG). After disruption of cells and centrifugation, the supernatant was subjected to chromatography on a Sulfate Cellulofine column (Seikagaku Co., Japan). Fractions containing FLAG-PI(4)P5K α were further subjected to chromatography on a column of anti-FLAG M2 affinity resins (Eastman Kodak Co.). Myr-ARF1 was expressed in *E. coli*, which was cotransfected with pBB131, with 0.4 mM IPTG in the presence of 0.2 mM myristic acid. Myr-ARF1 in the soluble fraction of the cell was purified by chromatography on a ProBond nickel-chelating column (Invitrogen) using 300 mM imidazole as an elution buffer. After removal of imidazole by dialysis, purified proteins were stored at -80°C until use.

Recombinant ARF1, RhoA, and Rac1 expressed in Sf9 cells were purified as described previously (Hammond et al., 1997).

Preparation of Bovine Brain Cytosol

Bovine brains were homogenized in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and centrifuged at 100,000 \times g for 30 min. The clear supernatant (4 mg/ml protein) was frozen in liquid nitrogen and stored at -80°C until use.

Purification of the GTP γ S-Dependent Activator of PI(4)P5K α from Bovine Brain Cytosol

Bovine brain cytosol proteins were precipitated with ammonium sulfate at 25%–60% saturation and then dissolved in 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1 mM DTT, and 0.1 mM PMSF (buffer A). After dialysis against buffer A, the sample was loaded onto a DEAE Sephacel column and eluted with a linear gradient of 0–0.4 M NaCl in buffer A. PI(4)P5K α -activating fractions eluted at 0.1 M NaCl were concentrated and then subjected to gel filtration chromatography on a Sephacryl S-300 HR column in buffer B which consisted of 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1 mM DTT, 0.1 mM PMSF, 1 μ M GDP, and 50 mM NaCl. PI(4)P5K α -activating fractions eluted were loaded onto a Hydroxyapatite column, and eluted with a linear gradient of 0–120 mM potassium phosphate (pH 7.5) in 20 mM Tris-HCl (pH 7.5), 0.1 mM DTT, 0.1 mM PMSF, and 1 μ M GDP. After an equal volume of 4 M ammonium sulfate was added to PI(4)P5K α -activating fractions eluted at 35 mM potassium phosphate, the fractions were further subjected to chromatography on a Phenyl-TOYOPEARL 650M column and eluted with a linear gradient of 2–0 M ammonium sulfate. PI(4)P5K α -activating fractions eluted at 0.6 M ammonium sulfate were concentrated and gel filtered on a Superdex 75 HR 10/30 column with buffer B.

Amino Acid Sequence Analysis of the Purified PI(4)P5K α Activator

The PI(4)P5K α activator purified from bovine brain cytosol (20 μ g of protein) was digested with 2 μ g/ml of trypsin at 37 $^{\circ}\text{C}$ overnight. The peptide fragments were separated by reversed-phase HPLC. The separated peptides were sequenced by a model PPSQ-21 sequencer (Shimadzu, Kyoto, Japan).

Assay of PI(4)P5K Activity

PI(4)P5K activity was determined by the incorporation of ³²Pi from [γ -³²P]ATP into PI(4)P. In a standard assay, samples containing PI(4)P5K were incubated at 37 $^{\circ}\text{C}$ for 25 min in 50 mM Tris-HCl (pH

7.5), 1 mM EGTA, 10 mM MgCl₂, 0.004% (w/v) NP-40, 50 μM PI(4)P, 50 μM [³²P]ATP (1 μCi/assay), either of 40 μM GTPγS or GDP, and the indicated concentrations of PA. For purification of a GTPγS-dependent PI(4)P5Kα activator, recombinant PI(4)P5Kα purified from COS-7 cells was reconstituted with each fraction separated by the column chromatographies in the presence of 0.2 M ammonium sulfate. After the reaction was terminated by adding chloroform/methanol/11.7 N HCl (200:400:1, by volume), lipids were extracted according to the methods of Bligh and Dyer (1959), and separated by thin layer chromatography on Merck silica gel 60 plates that were impregnated with 1.2% potassium oxalate in the solvent system of chloroform/methanol/acetone/water/acetic acid (7:5:2:2:2, by volume). The [³²P]PI(4,5)P₂ produced was analyzed by a BAS2000 Bioimaging analyzer (Fuji Photo Film, Tokyo, Japan).

Confocal Immunofluorescence Microscopy

HeLa cells grown in Dulbecco's modified Eagles medium (DMEM) supplemented with 5% fetal calf serum (FCS) on glass coverslips were transfected using Lipofectamine Plus (Life Technologies, MD). Transfected cells were treated as described in figure legends, then fixed, permeabilized, and blocked as previously reported (Torii et al., 1995). To stain PI(4,5)P₂ with the anti-PI(4,5)P₂ antibody (AM212), 0.01% Triton-X 100 was employed for permeabilization. The cells were then incubated with monoclonal anti-myc (9E10), anti-HA antibodies, and anti-PI(4,5)P₂ antibodies, followed by fluorescent staining with FITC- and Cy3-labeled secondary antibodies and Alexa 488 phalloidin. After being washed with PBS, the immunofluorescently stained cells were imaged using a laser-scanning confocal microscope (TCS4D, Leica Laser Technik).

[³⁵S]GTPγS Binding to Recombinant myr-ARF1

Recombinant myr-ARF1 was incubated for 25 min at 37°C with 10 μM [³⁵S]GTPγS in 50 mM Tris-HCl (pH 7.5), 1 mM EGTA, 10 mM MgCl₂, 0.004% NP-40, 12.5 μM PI(4)P, and 12.5 μM of the indicated phospholipids. After the reaction was terminated by adding 50 mM Na-HEPES (pH 7.4), 2.5 mM EDTA, 5 mM MgCl₂, and 40 μM GTP, samples were transferred to nitrocellulose filters. Filters were washed thoroughly, then the bound [³⁵S]GTPγS was quantified by liquid scintillation spectroscopy.

Western Blotting

Western blotting experiments were performed as described previously (Yamazaki et al., 1999) using ECL (Amersham).

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