Type I Phosphatidylinositol 4-Phosphate 5-Kinase Directly Interacts with ADP-ribosylation Factor 1 and Is Responsible for Phosphatidylinositol 4,5-Bisphosphate Synthesis in the Golgi Compartment*

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Phosphatidylinositol (PtdIns) 4,5-bisphosphate is involved in many aspects of membrane traffic, but the regulation of its synthesis is only partially understood. Golgi membranes contain PI 4-kinase activity and a pool of phosphatidylinositol phosphate (PIP), which is further increased by ADP-ribosylation factor 1 (ARF1). COS7 cells were transfected with α and β forms of PI 4-kinase, and only membranes from COS7 cells transfected with PI 4-kinase β increased their content of PIP when incubated with ARF1. PtdIns(4,5)P2 content in Golgi membranes was nonexistent but could be increased to a small extent upon adding either cytosol or Type I or Type II PIP kinases. However, when ARF1 was present, PtdIns(4,5)P2 levels increased dramatically when membranes were incubated in the presence of cytosol or Type I, but not Type II, PIP kinase. To examine whether ARF1 could directly activate Type I PIP 5-kinase, we used an in vitro assay consisting of phatidylic-containing liposomes, ARF1, and PIP 5-kinase. ARF1 increased Type I PIP 5-kinase activity in a guanine nucleotide-dependent manner, identifying this enzyme as a direct effector for ARF1.

Phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) has many functions in cells, both in signaling and in membrane traffic (1, 2). It is a substrate for phospholipase C and phosphoinositide 3-kinase, which leads to the production of second messengers. Additional functions require the intact lipid, and here it mediates the recruitment of proteins containing appropriate PIP2 recognition domains, which include proteins with PH domains and many cytoskeletal proteins (2). Many enzymes involved in the synthesis of phosphoinositides have been identified; these include PI 4-kinases α and β, which are localized at the Golgi, and two families of PIP kinases, which are mainly cytosolic (3–5). Although the PIP kinases were previously termed Type I and Type II PtdIns(4)P 5-kinases, recent studies have revealed that the two families selectively phosphorylate different positions on the inositol ring (6). Type I phosphorylates PtdIns(4)P at the 5-position to make PtdIns(4,5)P2, and Type II can phosphorylate PtdIns(5)P and PtdIns(3)P at the 4-position to make PtdIns(4,5)P2 and PtdIns(3,4,5)P3, respectively. Thus, two pathways for the synthesis of PtdIns(4,5)P2 can be identified (6): PI can be phosphorylated by PI 4-kinase to PtdIns(4)P and subsequently to PtdIns(4,5)P2 by Type I PIP 5-kinase. An alternative pathway is via phosphorylation of PI to PtdIns(5)P and subsequent phosphorylation by a Type II PIP 4-kinase (see Fig. 6). The enzyme responsible for making PtdIns(5)P could be PIKfyve (7), although a recent study identifies PIKfyve as a Type III PtdIns(3)P 5-kinase, which specifically phosphorylates PtdIns(3)P to make PtdIns(3,5)P2 (8).

The Golgi apparatus is an integral component of the secretory pathway. ADP-ribosylation factor 1 (ARF1) is a small G protein that is localized to the Golgi and is required for Golgi structure and function. One well established function of membrane-bound ARF1 is to recruit a complex of cytosolic proteins collectively known as coatomers, which, upon oligomerization, result in the formation of "COP1"-coated vesicles. Many downstream effectors of ARF1 have been identified; these include a PIP2-dependent phospholipase D (9, 10), arfaptin (11), and the β-subunit of coatomer (12). ARF1 has also been shown to regulate PIP2 levels in cytosol-depleted permeabilized cells (13, 14) as well as in Golgi-enriched fractions (15, 16). In vitro, Type I PIP 5-kinase can be activated by PA (17), which is the product of phospholipase D. It has therefore been suggested that PIP2 synthesis by ARF1 is indirect and is dependent on phospholipase D activation (13, 14). However, recent studies suggest that ARF1 can stimulate PtdIns(4,5)P2 synthesis by mediating the recruitment of PI 4-kinase β and an unidentified PIP 5-kinase from the cytosol to the Golgi complex (15). This ability to stimulate PIP2 synthesis was apparently independent of its activities on coat proteins and PLD (15).

The relationship between ARF and PIP2 is complicated. In addition to stimulating the synthesis of PIP2, ARF1 can directly interact with PIP2 (18) as can the ARF exchange factors and ARF-GTPase activating protein (GAP). Thus, ARNO, the exchange factor for ARF1, has a PH domain that is recognized by PIP2 (19), and over-expression results in disruption of the

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1 The abbreviations used are: PtdIns, phosphatidylinositol; ARF1, ADP-ribosylation factor 1; GAP, GTPase activating protein; PIP, phosphatidylinositol phosphate; PIP2, phosphatidylinositol bisphosphate; ARNO, ARF nucleotide-binding site opener; PA, phosphatidic acid; PLD, phospholipase D; PI 4-kinase, phosphatidylinositol 4-kinase; PC, phosphatidylcholine; GTP-S, guanosine 5’-3-O-thiophosphate; BSA, bovine serum albumin; TLC, thin layer chromatography; HPLC, high pressure liquid chromatography.
ARF-stimulated PIP 5-Kinase

Determination of Polyphosphoinositide Production in Golgi Membranes—ARF1 was recruited to Golgi membranes (2 μg/assay tube) by incubating ARF1 (10 μM) and GTP•S (20 μM) in Buffer A (25 mM Hepes, 25 mM NaCl, 2.5 mM MgCl₂, 0.2 mM succrose, 1 mM dithiothreitol, 1 mM MgATP, 1.6 mg/ml creatine phosphate, and 100 μg/ml creatine kinase, pH 7.2) for 15 min at 37 °C in an assay volume of 200 μl. After centrifugation at 1,000 g for 10 min at 4 °C and layering over a 1.3 M sucrose cushion and centrifuged at 200,000 × g for 1 h. The primary interface was recovered (Fig. 5A), and 1.5 volumes of 2 M sucrose were added. This was transferred to a centrifuge tube, overlaid with 1.1, 0.85, and 0.25 M sucrose, and centrifuged at 200,000 × g for 2 h. Three fractions (F1, F2, and F3) were collected and assayed for galactosyltransferase and ARF-stimulated PLD activity as described previously (24) (see Fig. 5A). F1 was highly enriched in galactosyltransferase and is referred to collectively as the Golgi membranes. Cytosol was obtained from pig brain and was kept at −80 °C until required. In brief, one liver was homogenized in 6 volumes of 250 mM sucrose in 20 mM Tris, pH 7.4, 1 mM EGTA, and a mixture of protease inhibitors. The post-nuclear supernatant was obtained after centrifugation at 1,000 × g for 10 min at 4 °C and subsequently deacylated by monomethylamine treatment; the glycerol backbone was removed exactly as described (23). The resulting supernatant was then centrifuged at 120,000 × g for 45 min to separate the membrane and cytosol. Phosphoinositide production was determined as described for Golgi membranes, except that COS7 cell membranes were present at 10 μg/assay tube.

In Vitro Assay for Measuring ARF-Stimulated PIP 5-Kinase Activity—Activity was analyzed in 100 μl of Buffer A (but without creatine phosphate and creatine kinase). Lipid vesicles were prepared by sonication to give final concentrations of 80 μg/ml phosphatidylcholine and 8 μg/ml phosphatidylinositol 4-phosphate in the final assay. When the effect of PA was examined, it was included in the pre-sonication mix so as to give 8 μM/ml in the final assay. All samples also contained 1 mg/ml BSA, 20 μg/ml recombinant rat ARNO, 2 μCi of [32P]ATP as well as 10 μM ARF1 and GTP•S (20 μM). 250 ng of Type I PIP 2 kinase was added, and the tubes were incubated at 30 °C for 20 min unless indicated otherwise. At the end of this time, samples were quenched with chloroform:methanol, and the production of PIP 2 was analyzed by TLC. GTP•S binding to ARF1 was measured under identical assay conditions to those described above, except radiolabeled ATP was omitted and [32P]-labeled GTP•S (1 μCi) was included. Only 4% of the ARF was GTP•S-loaded, and thus the concentration of active ARF is calculated as 400 nM.

RESULTS AND DISCUSSION

We used Golgi membranes from rat liver that have previously been shown to be enriched in PI kinase activity but are devoid of activity that makes PIP 2 (23). Golgi membranes were pre-incubated with ARF1 and GTP•S for 15 min to recruit ARF1 to membranes and were subsequently tested for PIP formation. PIP levels in these membranes were nearly double that found in membranes treated with an irrelevant protein, with no production of PIP 2 (Fig. 1). When cytosol is also included during the kinase reaction, PIP 2 production is greatly facilitated. We used Golgi membranes from rat liver that have previously been shown to be enriched in PI kinase activity but are devoid of activity that makes PIP 2 (23). Golgi membranes were pre-incubated with ARF1 and GTP•S for 15 min to recruit ARF1 to membranes and were subsequently tested for PIP formation. PIP levels in these membranes were nearly double that found in membranes treated with a control protein, with no production of PIP 2 (Fig. 1). When cytosol is also included during the kinase reaction, PIP 2 production is greatly facilitated in membranes pretreated with ARF1/GTP•S compared with no pretreatment or with GTP•S alone. The production of PIP only in the presence of cytosol implies that cytosol is the source of the PIP kinases. These data using cytosol as a source of the kinases are similar to those reported by Godi et al. (15), who concluded that ARF1 stimulates the synthesis of PIP 2 in Golgi membranes.

To examine whether the increase in PIP was due to increased PI 4-kinase activity rather than recruitment, we replaced the cytosol with purified PIP kinases. The addition of Type I or Type II PIP kinases increased the level of PIP 2 to a small extent in the untreated Golgi membranes. When Golgi membranes were incubated with ARF1 and GTP•S for 15 min, re-isolated, and incubated with Type I or Type II PIP kinases,
**ARF-stimulated PIP 5-Kinase**

PIPC levels increased considerably in the presence of Type I, but not Type II, PIP 5-kinase. (Fig. 1).

To analyze the PIP2 produced by Type I and Type II enzymes, the assay was scaled up. ARF1-pretreated Golgi membranes were incubated with either cytosol, Type I, or Type II PIP kinases, and the PIP2 formed was isolated by TLC. For the experiment using Type II, the scale-up factor had to be much greater, using a higher specific activity of ATP, to obtain sufficient counts (cpm) for the analysis. The PIP2 was separated by TLC, deacylated, and deglycerated to remove the fatty acids and the glycerol backbone. The inositol triphosphates were analyzed by HPLC as described previously (29). a, Ins(1,4,5)P3; and Ins(1,3,4)P3 standards. Golgi membranes were analyzed after incubation with cytosol (b), Type I PIP 5-kinase (c), and Type II PIP 4-kinase (d).

**Fig. 2.** HPLC analysis of the PIP2 formed in Golgi membranes with cytosol, Type I PIP 5-kinase, and Type II PIP 4-kinase. The Golgi membranes were pretreated with ARF1 as described for Fig. 1, except the incubations were scaled up to obtain sufficient counts (cpm) for the analysis. The PIP2 was separated by TLC, deacylated, and deglycerated to remove the fatty acids and the glycerol backbone. The inositol triphosphates were analyzed by HPLC as described previously (29). a, Ins(1,4,5)P3; and Ins(1,3,4)P3 standards. Golgi membranes were analyzed after incubation with cytosol (b), Type I PIP 5-kinase (c), and Type II PIP 4-kinase (d).

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PtdIns(5)P has only recently been identified as a minor component of mammalian cells (6). Our results indicate that Golgi membranes contain a pool of PtdIns(5)P and also a smaller pool of PtdIns(3)P that is available for conversion to PtdIns(4,5)P2 and PtdIns(3,4)P2. Alternatively, the relatively smaller size of the PtdIns(3,4)P2 could be because the Type II enzyme is less active toward PtdIns(3)P. Phosphorylation of these minor lipids by Type II PIP 4-kinase is not regulated by ARF1 treatment. In contrast, ARF1 pretreatment leads to an increase in PtdIns(4,5)P2 levels, which is derived from PtdIns(4)P phosphorylation by Type I PIP 5-kinase (Fig. 1). ARF1 pretreatment of Golgi has two effects: it increases the activity of an endogenous PI 4-kinase, thus increasing PIP levels, and it causes a dramatic increase in PIP2 levels.

To identify which PI 4-kinase was regulated by ARF1, we next transfected COS7 cells with FLAG-tagged PI 4-kinase α and β and prepared a crude membrane fraction. PI 4-kinase β was found in both the cytosol and membrane fractions, whereas PI 4-kinase α was mainly membrane-bound. Membranes were examined for ARF1-stimulated PI 4-kinase activity. Despite the expression of PI 4-kinases in the transfected cells (Fig. 3), the incorporation of label into PIP under basal activity was not very different than in untransfected cells. This finding is not surprising, because the p55 enzyme that remains uncloned probably accounts for the bulk of PI 4-kinase activity in most cells. We were unable to show a stimulation of PI 4-kinase activity in intact ARF1-pretreated membranes (data not shown). To observe whether ARF1 could directly regulate the PI 4-kinase β activity, we immunoprecipitated the PI 4-kinase β from the cytosol using the FLAG-tag and incubated the immunoprecipitate with PI/PC (1:10) vesicles in the presence of ARF1, ARNO and GTPγS. We were unable to show a stimulation of PI 4-kinase β activity by ARF1 in vitro (results not shown); this could be because of incorrect assay conditions or the effect of ARF1 on PI 4-kinase β is indirect.

Only in ARF1-pretreated membranes was the Type I PIP 5-kinase able to increase the PIP2 content, despite the presence of PIP in the membranes. The increase in PIP2 stimulated by ARF1 is disproportionate to the increase seen with PIP, suggesting that the increase in PIP2 is not entirely due to the
increase in PIP levels. This suggested that ARF1 could be having a direct effect on PIP 5-kinase. We developed an in vitro assay using PC vesicles with 10% PIP to examine this possibility. ARF1, ARNO, and GTPγS were added to these vesicles followed by Type I PIP 5-kinase in the presence of MgATP. ARF1 was able to stimulate PIP 5-kinase activity only when GTPγS or GTP was present (Fig. 4A). The increase in PIP₂ was dependent on the amount of ARF1 added to the assay (Fig. 4B). Furthermore, we also purified native ARF proteins from brain cytosol (10), which was also active in stimulating PIP 5-kinase activity (data not shown).

Honda et al. (32) have recently reported that PIP 5-kinase can also be activated by ARF1 but that this required the presence of PA. In these assay conditions, 50 μM PtdIns(4)P (10:1) was incubated with ARNO, ARF1, and GTPγS (20 μM) or GTP (250 μM) as indicated. Type I enzyme was added where indicated together with 1 μM 32P-labeled MgATP. After incubation for 20 min, samples were analyzed for PIP 2 production. A, ARF1 in the presence of GTPγS or GTP stimulates PIP 5-kinase activity; B, activation of PIP 5-kinase activity is dependent on ARF1 concentration. The active concentration of ARF1 was measured using GTPγS binding as described under “Experimental Procedures.” C, effect of PA on ARF1-stimulated PIP 5-kinase activity in vesicles containing PC. Vesicles were composed of PC:PIP:PA (10:1:1). D, effect of PA on ARF1-stimulated PIP 5-kinase activity in vesicles containing substrate alone or substrate with PA (PI(2):PA (1:1)).

From our data, we conclude that ARF can directly activate PIP 5-kinase and that PLD-derived PA is not required. This conclusion is supported by three observations. In the in vitro assay used here, PA alone does not activate PIP 5-kinase and potently inhibits ARF1-stimulated PIP 5-kinase activity. Furthermore, Golgi membranes used here that show ARF-stimulated PIP₂ production have only minimal ARF-stimulated PLD activity. Finally, butanol (which would remove any PA derived from PLD activity) is without effect on PIP₂ production.

Whether ARF1 directly regulates PIP 5-kinase β still remains to be established. Fig. 6 summarizes the pathways that are

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**Fig. 4. ARF1 directly stimulates Type I PIP 5-kinase activity in vitro.** Vesicles consisting of PC-PtdIns(4)P (10:1) were incubated with ARNO, ARF1, and GTPγS (20 μM) or GTP (250 μM) as indicated. Type I enzyme was added where indicated together with 1 μM 32P-labeled MgATP. After incubation for 20 min, samples were analyzed for PIP₂ production. A, ARF1 in the presence of GTPγS or GTP stimulates PIP 5-kinase activity; B, activation of PIP 5-kinase activity is dependent on ARF1 concentration. The active concentration of ARF1 was measured using GTPγS binding as described under “Experimental Procedures.” C, effect of PA on ARF1-stimulated PIP 5-kinase activity in vesicles containing PC. Vesicles were composed of PC:PIP:PA (10:1:1). D, effect of PA on ARF1-stimulated PIP 5-kinase activity in vesicles containing substrate alone or substrate with PA (PI(2):PA (1:1)).

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**Fig. 5. Dependence of ARF1-stimulated PIP₂ synthesis on PLD activity at Golgi membranes.** A, a purification of Golgi-enriched membranes. The schematic shows the preparation of the primary interface as well as fractions F1, F2, and F3. B, galactosyltransferase activity of 0.5 μg of membrane protein of each fraction. C, ARF1-stimulated PLD activity of each membrane fraction and is expressed as a percentage of the basal activity determined in the absence of ARF and GTPγS. Samples contained 5 μg of membrane protein, 10 μM ARF, and 20 μM GTPγS where added. D, effect of butanol and butan-2-ol on PIP₂ production in Golgi membranes. Butanol or butan-2-ol was added at 0.5%.

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**Fig. 6. The metabolic pathways that are present at the Golgi to account for the synthesis of polyphosphoinositides.** The ARF-regulated pathway is indicated by black arrows.
likely to be operative at the Golgi and indicates the reactions that are regulated by ARF1. The ability of ARF1 to stimulate an increase in PtdIns(4,5)P₂ levels suggests that this lipid has a specific function at this location. A potential function of PIP₂ at the Golgi could be in the recruitment of PIP₂-binding proteins, which could include spectrin (16)- and oxysterol-binding proteins (33), both of which have motifs that recognize PIP₂. In addition, PIP₂ may also regulate the ARF1 function via its interactions with ARNO, ARF1, and ARF-GAP. Phosphoinositides are also required for secretion from yeast Golgi (34, 35), and the yeast PI 4-kinase, Pik1 has been implicated in this.

Moreover, it is interesting to note that strains containing a Pik1 mutation exhibit synthetic lethality with mutant arf1 alleles (35). PIP₂ could play a similar function in secretion in the mammalian Golgi. Although the precise requirement for PIP₂ at the Golgi still needs to be clarified, it is clear that the need to critically control the levels of PIP₂ at this location is essential. The levels of PIP₂ at the Golgi are kept in check by a specific function at this location. A potential function of PIP₂ is that they are regulated by ARF1. The ability of ARF1 to stimulate an increase in PtdIns(4,5)P₂ levels suggests that this lipid has a specific function at this location. A potential function of PIP₂ at the Golgi could be in the recruitment of PIP₂-binding proteins, which could include spectrin (16)- and oxysterol-binding proteins (33), both of which have motifs that recognize PIP₂. In addition, PIP₂ may also regulate the ARF1 function via its interactions with ARNO, ARF1, and ARF-GAP. Phosphoinositides are also required for secretion from yeast Golgi (34, 35), and the yeast PI 4-kinase, Pik1 has been implicated in this.

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