Activation of Phospholipase D and Phosphatidylinositol 4-Phosphate 5-Kinase in HL60 Membranes Is Mediated by Endogenous Arf but Not Rho*

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Membrane-associated phospholipase D (PLD) in HL60 cells can be activated by the small GTP-binding proteins Arf and RhoA, but polyphosphorylated inositol lipids were required for maximum activity. The intact lipid was required because neither inositol 1,4,5-trisphosphate nor stearoyl-arachidonyl glycerol could substitute for phosphatidylinositol 4,5-bisphosphate (PIP2). Arf-stimulated but not Rho-stimulated PLD activity was increased by the inclusion of Mg2+ and ATP. ATP-dependent PLD activation occurred when phosphatidylinositol 4-phosphate (PIP), PIP2, or phosphatidylinositol 4,5-bisphosphate (PIP3) were included, but PIP2 formation was only detected with PIP; no PIP2 production was detected under any conditions. Therefore, the ATP-dependent increase in PLD activity cannot be explained by PIP2 or PIP3 formation. Association of endogenous Arf and RhoA with membranes was increased by incubation with GTPγS. This treatment increased membrane PLD and PIP kinase activities in the absence of exogenous P21 proteins. Reduction of Arf translocation suppressed the increase in PLD and PIP kinase activities, whereas complete removal of Rho but not Arf from membranes with RhoGDI was without effect on PLD activity but increased PIP kinase activity. Therefore, although recombinant Arf and Rho can activate PLD and PIP kinase in HL60 cells, it is the endogenous Arf but not Rho that regulates PLD, and thus a role for Rho in the physiological regulation of PLD in HL60 cells is unlikely.

Agonist-activated phospholipase D (PLD) activity is an important step in signal transduction. The product of PLD activity, phosphatidate, can be degraded to diacylglycerol by phosphatidate phosphohydrolase or be metabolized by phospholipases of the A class to lysophosphatidate and fatty acid. These lipids have all been proposed to function as signaling molecules. For example, diacylglycerol and unsaturated fatty acids are activators of most forms of the protein kinase C isoenzymes, and arachidonate is the precursor of leukotrienes and thromboxanes, mediators of inflammatory responses. The actions of phosphatidate are more complex because it has been suggested to function as an agonist and can regulate the activity of several intracellular proteins (for a detailed list see Ref. 1). Lysophosphatidate functions as an extracellular agonist rather than an intracellular agent.

The regulation of stimulated PLD activity is multifactorial, with protein kinase Cs, tyrosine kinases, and G-proteins all being suggested as regulators in various systems (2, 3). PLD does not appear to be directly receptor-coupled, and recently a role for small G-proteins in its regulation has been proposed (4, 5). Arf, first identified as the protein responsible for enhancing the cholera toxin catalyzed ADP-ribosylation of the Gsα subunit, has a key role in vesicular transport and has been shown to activate membrane bound PLD in a GTP-dependent manner in HL60 membranes.

The small G-protein RhoA has also been proposed to be a regulator of PLD activity (6). This protein is found predominantly in the cytosol of unstimulated cells in its GDP bound state associated with RhoGDI. The exact sequence of events remains ill defined, but RhoA binds to membranes in its GTP-bound state following disassociation from GDP dissociation inhibitor (7). RhoA can be inactivated by incubation with the Clostridium botulinum C3 toxin, which ADP-ribosylates RhoA in the putative effector domain on Asn14 (8). This inhibition has been used to identify RhoA-mediated events, such as stress fiber formation, focal adhesion complex formation, and recently PIP kinase activation. However, it has been reported that C3 treatment of liver plasma membranes did not inhibit a RhoA-regulated PLD activity (6).

The activation of PLD by Arf or RhoA is strongly dependent upon the presence of polyphosphoinositides, and it has been considered possible that the availability of PIP2 (4) or PIP3 (9) is an important factor in PLD activation. Because RhoA has been reported to activate PIP kinase (10), thereby increasing PIP2 biosynthesis, it is possible that this stimulation is the means of PLD activation. However, a recent report has shown that Rac but not RhoA interacts with and activates the type II isoform of PIP kinase, questioning the physiological role of RhoA (11).

We have investigated the activation of PLD by both Arf and RhoA in the presence of acidic lipids with or without ATP in the incubations to identify the potential role of PIP kinase or other kinases in PLD activation. Furthermore, by manipulating the
localization of Arf and RhoA under various conditions and relating the redistribution of these proteins to PLD and PIP kinase activation in the same membranes, we have assessed the relative physiological roles of the GTP-binding proteins in PLD and PIP kinase activation.

**EXPERIMENTAL PROCEDURES**

**Materials**—RPMI 1640 + Glutamax-I and heat-inactivated fetal calf serum were purchased from Life Technologies, Inc. phosphatidylethanolamine, PS, PIP2, phosphatidylphosphatidylcholine, Dowex (50W) hydrogen form, and glutathione were from Sigma. GTPγS, GDPγS, and ATP were from Boehringer Mannheim, and [γ-32P]ATP (AA0068, 3000 Ci/mmol), and Enhanced Chemiluminescence kit were from Amersham Corp. Glutathione-Sepharose was from Pharmacia Biotech Inc. High performance liquid chromatography aluminum silica gel 60 plates (5547) were from Merck. Coomassie protein assay reagent was from Bio-Rad. General chemicals (analytical grade) were from Sigma or BDH.

GST-RhoGDI expressed in Escherichia coli was generous gift from Dr. Alan Hall, University College London, UK. PIP2 was a generous gift from Dr. Roy Gigl, NIMR, Mill Hill, London, UK.

Antibodies against synthetic peptide sequences from Arf1 and 3 (amino acids 98–112) and RhoA (amino acids 87–103) were raised in sheep, and the IgG fraction of the supernatant was purified and used.

Preparation of Recombinant Proteins—Glutathione etiopoke tagged Arf was prepared from Sf9 cells using the method given previously for the cloning and purification of such proteins (12). GST-RhoA, expressed in E. coli, was prepared as given previously (13).

**Cell Culture and Fractionation**—HL60 cells were grown in RPMI + Glutamax medium supplemented with 15% (v/v) heat-inactivated fetal calf serum to a density of 1.0–1.2 × 10⁷/ml. Cells were harvested by centrifugation (800 g, 30 min) and washed three times using 137 mM NaCl, 8.1 mM Na2HPO4, pH 7.5, 2.7 mM KCl, 2.5 mM EDTA, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride (harvesting solution). Cells were sonicated and harvested solution was used for three 10-s bursts using a probe sonicator. Membrane and cytosolic fractions were prepared by centrifugation in a Beckman TLA 100.5 rotor at 435,000 × g for 20 min at 4 °C.

Translocation of G-proteins—Endogenously monomeric G-proteins from HL60 cytosol were translocated to membranes in the presence of 100 mM GTPγS according to the method of Bokoch et al. (7). Where indicated, Mg2+ EDTA or 2 mM recombinant RhoGDI were included in the incubation. Re-isolated membranes were washed in harvesting solution containing the same additions as the initial translocation and stored as aliquots at −70 °C until used.

**SDS-Polyacrylamide Gel Electrophoresis and Western Blotting**—SDS-polyacrylamide gel electrophoresis was performed using 12.5% gels. Following electrophoresis, membranes were probed with antibodies to RhoA and a common sequence from Arf1 and Arf3. Rho and Arf antibodies were used at 1:1000 and 1:3000, respectively. The proteins were visualized using the Amersham Enhanced Chemiluminescence kit.

**Enzyme Assays**—PLD assays were performed using the method of Brown et al. (4), with slight modifications. The reaction was carried out in a final volume of 50 μl. All assays contained 10 μg of membrane protein in harvesting buffer, 3 mM MgCl2, 2 mM CaCl2, guanine nucleotide (30 μM GTPγS or 2 μM GDPγS) and 5 μl of 4 mM NaCl, which increased the PLD activity (results not shown). Where indicated, 0.5 μg of Arf or RhoA, 2 mM MgATP (in 20 mM HEPS, pH 7.5), were included. The membranes and the above constituents in a volume of 30 μl were added to the whole mixture for 30 min at 37 °C, and the reaction was started by the addition of 20 μl of lipid substrate. The final concentration of lipids in the assay were 137 μM phosphatidylethanolamine, 8.6 μM phosphatidylinositol (specific activity, 58 Ci/mol, 25 nCi/assay) and where indicated 6 μM SAG or 50 μM PIP2 and unless indicated 12 μM PIP2 or 32 μM PIP3. When PIP2 or PIP3 were used, less than 12 μM, sufficient MgCl2 was added such that the combined calcium/lipid concentration was maintained at 12 μM. The dried lipids were sonicated using a probe sonicator in a buffer such that the final concentrations in the assay were 50 mM HEPS, pH 7.5, 3 mM EDTA, 80 mM KCl, and 1 mM dithiothreitol. The incubation was performed for 1 h at 37 °C and stopped by the addition 750 μl of methanol/chloroform (2:1, v/v). Phases were split with 250 μl of chloroform and 400 μl of water. After vortexing and centrifugation (2000 × g for 10 min), 600 μl of the chloroform/aqueous phase was taken, and the choline was separated using Dowex columns, as given previously (14).

PLD and PIP kinase assays were performed using essentially identical conditions to PLD except that 20 μg of membrane protein was employed and 2 μg [γ-32P]ATP (specific activity, 10 Ci/mol, 1 μCi/assay) was used in all assays. The reaction was initiated by the addition of lipid substrate and continued for 7 min at 37 °C. The reaction was stopped with 750 μl of methanol/chloroform/HCl (150:75:1, v/v/v), and the phases were split with 250 μl of chloroform and 400 μl of water. The upper phase containing the unreacted [γ-32P]ATP was removed, and the chloroform-rich phase was washed once with 1 ml of water. The organic phase was then dried under vacuum, and the lipids were spotted onto a Merck silica gel 60 aluminum 10-cm plate that had previously been soaked in 1% (w/v) potassium oxalate (in methanol/water, 3:2) and dried at 100 °C for 20 min. Authentic PIP2, PIP3, or PIP2 was spotted on each plate, and the lipids were separated using chloroform/acetone/methanol/acetic acid/water, (40:15:13:12:5, v/v/v/v/v) or when PIP2 was separated propan-1-ol (20:80, v/v) (65:35). The lipid standards were located using iodine vapor, and the [32P]lipids were identified and quantified using a Molecular Dynamics PhosphorImager.

Protein was assayed using the Bio-Rad protein assay. For each experiment means and ranges or standard deviations as appropriate are given. The number of separate experiments performed for each figure are given in the legends.

**RESULTS**

Regulation of Arf- and RhoA-stimulated PLD Activity by Acidic Lipids and ATP—Initial experiments confirmed that HL60 cell membranes contained a PLD activity that was stimulated by recombinant Arf in the presence of GTPγS (30 μM) but not GDPγS (2 μM). In agreement with previous reports this activity was also enhanced by the inclusion of a cytosolic fraction prepared from these cells (4). This cytosolic preparation contained negligible PLD activity (results not shown). The activity was dependent on the presence of the polyphosphoinositides PIP2 or PIP3. Fig. 1A shows that PIP2 (in the absence of polyphosphoinositide) did not support PLD activity and that over the range of 1–12 μM phospholipid PIP2 was a slightly more potent activator than PIP3, whereas PIP2 was a poor activator (Fig. 1B). The polyphosphoinositol-dependent PLD activity was increased in the presence of Mg2+ and ATP compared with Mg2+ alone (Fig. 1B). In the absence of GTPγS, ATP did not support PLD activity (data not shown).

Recombinant RhoA also activated the PLD in HL60 membranes in a GTPγS-dependent manner. The activation by RhoA was less potent than by Arf (Fig. 2), whereas in combination the activation was approximately additive. The inclusion of Mg2+ and ATP did not increase the RhoA-stimulated PLD activity to a greater extent than with PIP2 or PIP3 (Fig. 2) were employed.

Stimulated PIP2 hydrolysis precedes against activated PLD activity in many cells, the ability of the inositol trisphosphate head group and the diacylglycerol moieties of PIP2 to stimulate Arf-dependent PLD were examined. 6 μM SAG was unable to activate PLD. IP3 at 50 μM or 100 μM did not activate PLD either in the absence or the presence of SAG. IP3 also failed to function an inhibitor of PIP2-stimulated PLD (results not shown).

**Pip Kinase Activity and Its Relationship to PLD Activity**—The results given above demonstrated that Arf- and RhoA-stimulated PLD activity was dependent upon the presence of PIP2 or PIP3. The possibility that the increased PLD activity seen the presence of Arf (Fig. 1B) was the result of the GTP-binding proteins stimulating the production of polyphosphoinositides was investigated by measuring the incorporation of [32P] from [γ-32P]ATP into phosphoinositides. These experiments utilized conditions similar to the measurement of PLD activity, so that the PLD and PIP kinase assays were comparable. When PIP (50 μM) was employed, PIP kinase activity was increased by Arf (control activity of 85.5 ± 28.9 and plus Arf of 160.2 ± 18.9; pmol PIP2 formed/mg protein/min; means ± S.D. for seven separate experiments min; taken from Fig. 3A and control membranes in Figs. 4C and 5C). However, no consistent
The effect upon kinase activity was observed in response to RhoA, with two experiments showing a small increase and two showing a small decrease in [32P]PIP2 formation (see control membranes in Fig. 5C). When the PIP was replaced by 12 μM PIP2 or 12 μM PIP3, the [32P] incorporation into PIP2 was lower than that seen with PIP and not stimulated by Arf (Fig. 3A). Furthermore, the incorporation of phosphate into PIP3 was considerably lower than that into PIP2 in the presence of the absence of Arf using PIP, PIP2, or PIP3 (Fig. 3B).

The Effect of Membrane Translocation of Endogenous Arf and RhoA upon PLD and PIP Kinase Activities—The experiments described above utilized exogenous recombinant GTP-binding proteins; it was therefore considered important to examine regulation of PLD activity by the endogenous RhoA and Arf proteins. The intracellular location of RhoA and Arf was modulated as described by Bokoch et al. (7). Translocation of endogenous Arf and RhoA from the cytosol to membranes was assessed using specific antibodies against each of the G-proteins. Figs. 4A and 5A show that the inclusion of 100 μM GTPγS in incubations of membranes and cytosol induced a translocation of both endogenous Arf and RhoA to the membranes. However, when 10 mM EDTA was also included in the incubations, the translocation of Arf was decreased, whereas that of RhoA was clearly increased (Fig. 4A). The membranes re-isolated from these incubations were used in assays for PLD and PIP kinase. Control incubations were performed in the absence of GTPγS or EDTA, and the membranes were isolated.

When assayed in the absence of exogenous Arf, the PLD activity in the membranes that had been pre-incubated with GTPγS (80 ± 15.9 pmol choline liberated/mg protein/min; mean ± S.D. of six separate experiments) was increased by an average of 2.5-fold compared with control (32.8 ± 12.8 pmol choline liberated/mg protein/min; mean ± S.D. of six separate experiments) membranes that had been pre-incubated in the absence of GTPγS. When EDTA was included in the pre-incubation, the increase was 1.8-fold (54.6 ± 19.4 pmol choline liberated/mg protein/min; mean ± S.D. of six separate experiments). This lower PLD activity was not due to loss of PLD protein from the membranes because the inclusion of recombinant Arf in assays of the membranes from the GTPγS/EDTA and control pre-incubations produced equivalent choline generation (Fig. 4B).
In the same samples a small but consistent increase in PIP kinase activity of 1.2-fold was observed in the membranes pre-incubated with GTPγS (135.6 ± 12.1 pmol PIP2 formed/mg protein/min; mean ± S.D. of three separate experiments compared with control incubations (112.5 ± 9.4 pmol PIP2 formed/mg protein/min; mean ± S.D. of three separate experiments), whereas a decrease to 0.76-fold (84.0 ± 10.3 pmol PIP2 formed/mg protein/min; mean ± S.D. of three separate experiments) was observed in membranes where EDTA had been included in the pre-incubation. This decrease could be due to loss of PIP kinase protein from the membranes because Arf failed to increase the PIP2 production to levels observed in membranes prepared in the absence of EDTA (Fig. 4C).

Membranes re-isolated from incubations containing control, GTPγS, or GTPγS plus RhoGDI were probed for Arf and RhoA.

**Fig. 3.** The effect of Arf on the phosphorylation of phosphatidylinositols using HL60 membranes. In addition to the standard assay components given under "Experimental Procedures," each incubation contained 20 μg of membrane protein, 2 mM ATP, 1 μCi of γ-32P ATP, 2 mM MgCl2, 30 μM GTPγS, and the indicated phosphatidylinositol. The open bars represent assays carried out in the absence of 0.5 μg of Arf, and the shaded bars represent assays carried out in the presence of 0.5 μg of Arf. The results given in A show the pmol of phosphate incorporated into PIP2, and those in B show the pmol of phosphate incorporated into PIP3. It was possible that more than 1 [32P]Pi was incorporated into lipids in some conditions (for example PIP2 when PIP was used in the assays), meaning that the specific radioactivity of the product could vary. Therefore, the results are expressed as pmol phosphate incorporated into lipid. The production of PIP3 from PIP given in A is taken as an assay of PIP kinase. The data were calculated by spotting a portion of the radioactive substrate onto the TLC plate prior to phosphorimaging. The image units produced by the known quantity in this sample was used to calculate the pmol of phosphate incorporated. The results shown are the means ± ranges of two independent experiments.

In the same samples a small but consistent increase in PIP kinase activity of 1.2-fold was observed in the membranes pre-incubated with GTPγS (135.6 ± 12.1 pmol PIP2 formed/mg protein/min; mean ± S.D. of three separate experiments compared with control incubations (112.5 ± 9.4 pmol PIP2 formed/mg protein/min; mean ± S.D. of three separate experiments), whereas a decrease to 0.76-fold (84.0 ± 10.3 pmol PIP2 formed/mg protein/min; mean ± S.D. of three separate experiments) was observed in membranes where EDTA had been included in the pre-incubation. This decrease could be due to loss of PIP kinase protein from the membranes because Arf failed to increase the PIP2 production to levels observed in membranes prepared in the absence of EDTA (Fig. 4C).

Membranes re-isolated from incubations containing control, GTPγS, or GTPγS plus RhoGDI were probed for Arf and RhoA.

**Fig. 4.** A, the effect of GTPγS and EDTA on the association of endogenous Arf and Rho with HL60 membranes. Membranes were re-isolated following a 10-min incubation at 30°C with cytosol at a protein ratio of 3:1 cytosol/membranes in a control incubation (lane 1), in the presence of GTPγS (100 μM) (lane 2), EDTA (10 mM) plus GTPγS (100 μM) (lane 3), recombinant Arf (lane 4), and recombinant RhoA (lane 5). The proteins were detected using antibodies raised against specific amino acid sequences contained in Arf (upper blot) and Rho (lower blot), respectively. Similar results were obtained in a total of six independent experiments. Recombinant Arf and Rho were also blotted to demonstrate the specificity of the antibodies. Arf could be detected in the control membranes (lane 1) when the time of exposure of the film to the chemiluminescence was increased. The recombinant Arf runs slightly higher than the HL60 Arf because of the epitope tag, and a doublet was seen that corresponded to myristoylated and nonmyristoylated forms (16). B, phospholipase D activity in HL60 membranes loaded with differing amounts of endogenous Arf or Rho. Pre-isolated membranes from the translocation system described in A were assayed for PLD activity in the presence of GTPγS (30 μM) in the absence or the presence of exogenous Arf (0.5 μg). The results shown are the means ± S.D. of six separate experiments. C, phosphatidylinositol 4-phosphate 5-kinase activity in HL60 membranes loaded with differing amounts of endogenous Arf or Rho. The pre-loaded membranes (A) were assayed for PIP kinase in the presence of GTPγS (30 μM) in the absence or the presence of exogenous Arf (0.5 μg). The results are the means ± S.D. for three separate experiments.

The inclusion of RhoGDI completely extracted RhoA from the membranes without affecting the membrane-association of Arf (Fig. 5A). PLD and PIP kinase assays performed on these membranes are shown in Fig. 5 (B and C). The extraction of the RhoA had very little effect on the PLD activity in these samples.
Regulation of PLD and PIP Kinase by Arf and Rho

PIP kinase activity increased in membranes pre-incubated with GTPγS as reported above; however, GST-RhoGDI inclusion caused a further increase in the activity (GTPγS-preincubated membranes 104.7 ± 0.6 and GTPγS/RhoGDI-preincubated membranes 169.0 ± 20.6 pmol PIP2 formed/mg protein/min; means ± range of two independent experiments when assayed in the absence of exogenous p21 protein). PIP kinase activity in RhoGDI-treated membranes was higher than in the control membranes in both experiments under all assay conditions employed.

**DISCUSSION**

The results presented in this paper demonstrate that although both RhoA and Arf can activate PLD in HL60 membranes in a PIP2- and GTPγS-dependent manner, it is probable that only Arf is the physiologically relevant activator. Maximum GTP-regulated PLD activity was dependent upon the presence of polyphosphorylated inositol lipids (Fig. 1) and (4). In agreement with previous reports, PS was not a PLD activator, and PIP was a weak stimulator, whereas PIP2 and PIP3 were both potent activators. These data indicate that there was not a general stimulation of PLD activity by acidic lipids. PIP2 hydrolysis is frequently observed when PLD is stimulated in an intact cell, raising the possibility that it is actually one of the products of this reaction that is the physiological activator. Thus SAG, the lipid portion, and the water soluble head group, IP3, of PIP2 were added alone or in combination to PLD assays. This protocol was also employed in an attempt to define which moiety of the PIP2 molecule is the activator. The concentration of SAG employed (6 μM) was intended to represent a concentration that could be produced in actual assays. On the other hand, the concentration of IP3 used (50–100 μM) was higher than the PIP2 concentration because this metabolite is susceptible to hydrolysis catalyzed by membrane-associated inositol polyphosphate 5-phosphatase under the PLD assay conditions (15). In an attempt to minimize IP3 production, the concentration of Mg2+ in these assays was reduced to 1 mM (this decreased the PLD activity by approximately 50%). SAG and IP3 alone or in combination could neither replace PIP2 nor inhibit the PLD activity detected in the membrane or the enzyme itself or to an associated regulatory molecule. This binding is unlikely to be via a Pleckstrin Homology domain because it has been shown previously that IP3 blocks the binding of PIP2 to proteins containing such a domain (16).

The Arf-stimulated PLD activity was enhanced in the presence of ATP (Figs. 1B and 2). Because PIP can be phosphorylated to PIP2, it is possible that this increase was secondary to PIP kinase activation, as suggested by Pertile et al. (17). ATP also increased PLD activity in the presence of PIP2 or PIP3, raising the possibility that re-synthesis of PIP2 or PIP3 under the assay conditions was responsible for the increased PLD activity. This was investigated by measuring the incorporation of 32P from [γ-32P]ATP into PIP2 and PIP3 in the presence of PIP or PIP3 under the conditions employed in the PLD assays. When PIP was present [32P]PIP2 was produced, and Arf stimulated this activity (Fig. 3A). Therefore, the ATP-dependent increase in PLD activity seen under these conditions could be mediated by PIP kinase generated PIP2. However, when PIP2 or PIP3 were employed, little PIP2 was made, and no activation by Arf was seen. Furthermore, very little [32P]PIP3 was generated under any conditions tested here, demonstrating that the ATP-mediated increase in Arf-stimulated PLD activity in the presence of PIP2 or PIP3 was not the result of phosphoinositide phosphorylation. It is also unlikely that the...
conversion of PIP$_2$ to PIP$_3$ would result in a significant activation of PLD in this system because PIP$_3$ is only slightly more potent than PIP$_2$ as an activator (Fig. 1A). This suggests that the ATP was being utilized by a different kinase activity. The ATP-dependent increase in PLD activity was observed in the presence of Arf but not RhoA; thus the ATP effect upon PLD activity was probably on an Arf-mediated process rather than on PLD directly. A number of potential regulatory proteins such as Arf-GTPase-activating protein, Arf-guanine nucleotide exchange factor, or even Arf itself are targets of a putative regulatory kinase. The notion that a kinase other than PIP kinase is responsible for the increased PLD activity when Mg$^{2+}$/ATP and PIP$_2$ or PIP$_3$ were included in the assay also argues against the proposal that PIP$_2$ production was involved in Mg$^{2+}$/ATP plus PIP-stimulated PLD activation. Nevertheless, the inability of RhoA to activate PIP kinase in these cells (see Fig. 5C, control incubations) could explain why ATP did not increase PLD activity when RhoA was used as the activator and provides support for the proposal that PIP$_2$ production from PIP indeed activates PLD, unless the Rho regulatory proteins are not controlled by phosphorylation.

In an intact cell, stimulated translocation will be a fundamental point of regulation of any pathway regulated by small GTP-binding proteins. Thus the preparation of membranes containing variable levels of endogenous Arf and RhoA was an ideal system in which to test for regulation of enzyme activities by such proteins. A small but consistent increase in the activity of PIP kinase was detected in membranes loaded with both Arf and RhoA measured in the absence of exogenous Arf. This presumably reflects stimulation by the translocated proteins. The inclusion of 10 mM EDTA during translocation decreased the Arf, increased the RhoA membrane localization (Fig. 4A), and blocked the increase in PIP kinase activity (Fig. 4C). However, the PIP kinase activity measured in the presence of exogenous Arf was lower in the EDTA-treated membranes than the control membranes, indicating that the PIP kinase protein itself or an additional regulator had been lost from the membranes (Fig. 4C). The data in Figs. 3, 4C, and 5C showed that under control conditions PIP kinase was activated by exogenous Arf, and in Fig. 5C a small inhibition by exogenous RhoA was seen. Selective, complete blockage of RhoA, but not Arf, translocation with GST-RhoGDI increased the PIP kinase activity compared with RhoA-containing membranes. This was particularly surprising because it has been proposed that RhoA is an activator of PIP kinase (10). Although this has been questioned (11) and our data would also tend to argue against this proposal, inhibition has not been previously described. However, the addition of exogenous RhoA to the assays was without effect upon the PIP kinase activity in either the Arf- and RhoA-depleted or the Arf-loaded RhoA-depleted membranes (Fig. 5C). Thus RhoA was not acting as a direct regulator of PIP kinase in the Arf- and RhoA-loaded membranes. It seems most likely that the GST-RhoGDI removed another factor that was limiting the activity of PIP kinase in this system. Thus the role of Arf in PIP kinase regulation is not clear. However, in addition to the proposed function for this protein, and for Rac (11), in regulating PIP kinase, our results suggest that Arf may also play a role. It is unclear if this reflects differences in PIP kinase subtypes.

The PLD activity in membranes loaded with Arf and RhoA was activated by GTP$\gamma$S and PIP$_2$ in the absence of exogenous p21 protein. Reducing the translocation of Arf and increasing that of RhoA by the inclusion of EDTA in the pre-incubation, resulted in membranes in which stimulated PLD activity was decreased. However, in contrast to PIP kinase, these membranes did not contain less PLD because the addition of exogenous Arf stimulated PLD activity to an approximately equal extent in each group of membranes (Fig. 4B). The use of RhoGDI to prevent RhoA binding to membranes did not decrease the measurable PLD activity when compared with the Arf- and RhoA-loaded membranes (Fig. 5B). Therefore, the membrane PLD activity was dependent on the presence of Arf but not RhoA. Activation of PLD by exogenous (recombinant) RhoA was partially inhibited by Botulinum C3 toxin (results not shown), with no effect upon Arf-activated PLD, indicating that Arf did not require the presence of RhoA to activate PLD. Furthermore, the PLD activity in the Arf and RhoA loaded membranes was not inhibited by the C3 toxin (not shown), indicating that RhoA was not involved in the PLD activation in this system, in agreement with the data obtained with RhoGDI-treated membranes. The activation of membrane PLD by exogenous Rho has been previously reported (18), indeed it has been demonstrated that recombinant Rho and Arf activated partially purified porcine brain PLD synergistically (19). Treatment of liver membranes with GST-RhoGDI was shown to reduce basal PLD activity, which was partially restored by exogenous Rho (6). It was unclear what level of Arf was present in the treated membranes, which may be relevant because in the experiments reported here Arf could be detected. We have not observed synergy, as reported by Singer et al. (19), rather we detected additivity in the effects of submaximal exogenous Arf and Rho in the stimulation of PLD activity; thus it is possible that Rho can regulate PLD activity when the Arf concentration is limiting. Nevertheless, an increase in endogenous membrane Rho concomitant with a decreased Arf association (Fig. 4A) failed to maintain PLD activity (Fig. 4B). This suggests that endogenous Rho does not regulate PLD activity in H60 membranes. It is perhaps relevant that activation of partially purified phospholipase C by the G$_q$ and G$_\text{11}$ GTP-binding proteins was demonstrated (20) prior to the identification of G$_q$ and G$_\text{11}$ and that nonphysiological activation of the same phospholipase was detected when p21ras was over-expressed in NIH3T3 cells (21). Thus, under certain conditions it may be possible to activate phospholipases with inappropriate G-proteins. However in an intact cell, when the translocation of Arf is stimulated, as will occur upon receptor activation, PLD will be regulated by this G-protein. Therefore, whereas Rho and other small molecular weight G-proteins can function in an in vitro assay as PLD regulators, we believe that as originally suggested (4, 5), the physiological regulator of phospholipase D, at least in H60 membranes, is Arf.

**REFERENCES**

1. Martin, A., Gomez-Munoz, A., Duffy, P. A., and Brindley, D. N. (1994) Signal-Activated Phospholipases, pp. 139–164
2. Cook, S. J., and Wakelam, M. J. O. (1992) Rev. Physiol. Biochem. Pharmacol. 119, 14–45
3. Exton, J. H. (1994) Biochim. Biophys. Acta 1212, 26–42
4. Brown, A. H., Goto, S., Moomaw, C. R., Slaughter, C., and Sternweis, P. (1993) Cell 75, 1137–1144
5. Cockcroft, S., Thomas, G. M. H., Fensome, A., Geny, B., Cunningham, E., Guo, I., Hiles, I., Totty, N. F., Truong, O., and Husain, J. J. (1994) Science 263, 523–526
6. Malcolm, K. C., Ross, A. H., Qui, R., Symons, M., and Exton, J. H. (1994) J. Biol. Chem. 269, 25951–25954
7. Bokoch, G. M., Bohl, B. P., and Chuang, T.-H. (1994) J. Biol. Chem. 269, 31694–31679
8. Sekine, A., Fujigawa, M., and Narumiya, S. (1989) J. Biol. Chem. 264, 8602–8605
9. Lisovskikh, M., Chalifa, V., Pettle, P., Chen, C. S., and Cantley, L. C. (1994) J. Biol. Chem. 269, 21403–21406
10. Cheng, L. D., Traynor-Kaplan, A., Bokoch, G. M., and Schwartz, M. A. (1994) Cell 79, 507–513
11. Tolias, K. F., Cantley, L. C., and Carpenter, C. L. (1995) J. Biol. Chem. 270, 17660–17665
12. Portfiri, E., Evans, T., Chardin, P., and HNAD, J. F. (1994) J. Biol. Chem. 269, 22572–22577
13. Ridley, A. J., Paterson, H. F., Johnston, C. L., Diekmann, D., and Hall, A. (1992) Cell 70, 401–410
14. Cook, S. J., and Wakelam, M. J. O. (1989) Biochem. J. 263, 581–587
15. Hodgkin, M., Craxton, A., Parry, J. B., Hughes, P. J., Potter, B. V. L., Michell, R. H., and Kirk, C. J. (1994) Biochem. J. 297, 637–645
16. Harian, J. E., Hajduk, P. J., Yoon, H. S., and Fesik, S. W. (1994) Nature 371, 168–170
17. Pertile, P., Liscovitch, M., Chalifa, V., and Cantley, L. C. (1995) J. Biol. Chem 270, 5130–5135
18. Siddiqi, A. R., Smith, J. L., Ross, A. H., Qiu, R. G., Symons, M., and Exton, J. H. (1995) J. Biol. Chem 270, 8466–8473
19. Singer, W. D., Brown, H. A., Bokoch, G. M., and Sternweis, P. C. (1995) J. Biol. Chem 270, 14944–14950
20. Banno, Y., Nagao, S., Katada, T., Nagata, K., Ui, M., and Nozawa, Y. (1987) Biochem. Biophys. Res. Commun. 146, 861–869
21. Wakelam, M. J. O., Davies, S. A., Houslay, M. D., McKay, I., Marshall, C. J., and Hall, A. (1986) Nature 323, 173–176