ADP-ribosylation factor 1-regulated phospholipase D activity is localized at the plasma membrane and intracellular organelles in HL60 cells

Jacqueline WHATMORE*, Clive P. MORGAN*, Emer CUNNINGHAM*, Kate S. COLLISON*†‡, Keith R. WILLISON† and Shamsad COCKCROFT*§

*Department of Physiology, University College London, London WC1E 6JJ, and †CRC Centre for Cell and Molecular Biology, Institute of Cancer Research, Chester Beatty Laboratories, 237 Fulham Road, London SW3 6JB, U.K.

ADP-ribosylation factor (ARF), a small GTPase required for vesicle formation, has been identified as an activator of phospholipase D (PLD), thus implying that PLD is localized at intracellular organelles. HL60 cells were prelabelled with [^{14}C]acetate for 72 h and, after disruption, fractionated on a linear sucrose gradient. ARF1-regulated PLD activity in each fraction was assessed by measurement of phosphatidylethanol production. Two peaks of activity were identified, coincident with markers for Golgi/endoplasmic reticulum/granules (endomembranes) and plasma membrane respectively. Analysis of the fractions using exogenous phosphatidylcholine as substrate confirmed the presence of ARF1-dependent PLD activity in endomembranes and plasma membrane, and also identified an additional activity in the cytosol. In formyl-Met-Leu-Phe-stimulated cells, PLD activity as assessed by phosphatidylethanol formation was also associated with both the plasma membrane and endomembranes. Since ARF1-regulated PLD activity requires phosphatidylinositol 4,5-bisphosphate (PIP_2), the distributions of inositol lipids and the kinases responsible for lipid phosphorylation were examined. PIP_2 was highly enriched at the plasma membrane, whereas phosphatidylinositol (PI) and phosphatidylinositol 4-phosphate (PI4P), the precursors for PIP_2 synthesis, were found predominantly at endomembranes. The distribution of PI 4-kinase and PI4P 5-kinase activities confirmed the plasma membrane as the major site of PIP_2 production. However, endomembranes possessed substantial PI 4-kinase activity and some PI4P 5-kinase activity, illustrating the potential for PIP_2 synthesis. It is concluded that: (1) ARF1-regulated PLD activity is localized at endomembranes and the plasma membrane, (2) PIP_2 is available at both membrane compartments to function as a cofactor for ARF-regulated PLD, and (3) in intact cells, formyl-Met-Leu-Phe stimulates PLD activity at endomembranes as well as plasma membrane.

INTRODUCTION

In many cell types, including HL60 cells, it has been established that the non-hydrolysable analogue of GTP, guanosine 5′-[γ-thio]triphosphate (GTP[S]), is a potent activator of phospholipase D (PLD) activity [1–3]. PLD principally catalyses the hydrolysis of phosphatidylcholine (PC) to phosphatidic acid (PA) and free choline. Studies in HL60 cells have shown that the presence of both membranes and cytosol are necessary in order to observe GTP[S]-stimulated PLD activity [1,3,4]. Reconstitution studies using HL60 membranes or cytosol-depleted cells have led to the purification of several regulatory cytosolic factors, of which two have been identified as the highly related small GTP-binding proteins ADP-ribosylation factor 1 (ARF1) and ARF3 [5,6].

ARF proteins were originally identified as a cofactor for the ADP-ribosylation of the α-subunit of G_s by cholera toxin. A central role for ARF in constitutive secretion was established from biochemical studies which identified ARF as a component of protein coats. This factor was found associated with vesicles involved in protein transport between Golgi stacks [7–10]. The localization of ARF to the cytosolic side of the Golgi complex [7,11,12] has provided further evidence that this is a major site of ARF action. Additionally, ARF-GTPase-activating protein is also found specifically at the Golgi [13]. A role for ARF in regulated exocytosis has been recently identified in HL60 cells [14].

In addition to ARF, members of the Rho family of GTPases have also been shown to activate PLD in the presence of GTP[S] [15,16]. It was initially reported that Rho-guanine nucleotide dissociation inhibitor (GDI), a regulatory protein which specifically binds to and inhibits the functions of Rho proteins, inhibited GTP[S]-dependent PLD activity in neutrophils [15]. Subsequently, exogenously added RhoA was shown to reconstitute GTP[S]-stimulated PLD activity in Rho-GDI-treated rat liver plasma membranes [17] and HL60 cell membranes [16]. Rho proteins and ARF are thought to regulate PLD activity in a highly synergistic fashion [18,19].

Attempts to purify PLD have identified an additional requirement for phosphatidylinositol 4,5-bisphosphate (PIP_2) in the activation of the enzyme by small GTP-binding proteins. Brown et al. [6] demonstrated that, in an *in vitro* system, ARF regulation of the activity of a partially purified PLD was dependent on vesicles containing PIP_2. It has since become evident from studies with solubilized preparations from rat brain membranes that there are two chromatographically distinct forms

Abbreviations used: PLD, phospholipase D; PC, phosphatidylcholine; PEt, phosphatidylethanol; PI, phosphatidylinositol; PI4P, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PA, phosphatidate; GTP[S], guanosine 5′-[γ-thio]triphosphate; ARF, ADP-ribosylation factor; rARF1, recombinant ARF1; PE, phosphatidylethanolamine; PI-TP, PI transfer protein; FMLP, formyl-Met-Leu-Phe, HLA, human leucocyte antigen; GDI, guanine nucleotide dissociation inhibitor; β'-COP, β' coat protein.

‡ Present address: King Faisal Specialist Hospital and Research Centre, Department of Biological and Medical Research, MBC#03, P.O. Box 3354, Riyadh 11211, Kingdom of Saudi Arabia.

§ To whom correspondence should be addressed.

of PLD [20]. One form is regulated by ARF proteins and requires PIP_2 for activity, whereas a second, oleate-sensitive, form is not dependent on either ARF or PIP_2. Similarly, PIP_2 was not required in order to observe the activity of the oleate-sensitive PLD purified from pig lung [21]. An ARF-regulated mammalian PLD has been cloned from HeLa cells which is stimulated by PIP_2 and ARF [22].

Generally, PLD activity has been shown to be associated with membranes in mammalian cells [6,20,21], although some evidence has been reported that it may also be cytosolic [16,23]. Many cell surface receptors, when occupied by appropriate agonists, cause activation of PLD, which has led to the assumption that PLD activity is localized at the plasma membrane [24–26]. Rho proteins have been implicated in the muscarinic cholinergic activation of PLD in HEK cells transfected with the muscarinic receptor [27]. Inactivation of Rho was found to inhibit activation of PLD by the muscarinic cholinergic agonist in intact cells. However, a role for ARF in this process cannot be excluded if ARF involvement is downstream of Rho. In RBL cells, cross-linking of IgE receptors by antigen both stimulates PLD activity [28] and causes the association of ARF with the Golgi complex [12]. ARF proteins are associated with the Golgi [11], post trans-Golgi secretory vesicles [29] and clathrin-coated vesicles [30], suggesting that ARF-regulated PLD activity may be co-localized at these intracellular membranes. Ktistakis et al. [31] have recently reported the presence of ARF-stimulated PLD activity in Golgi-enriched membranes from several cell lines. The present investigation was undertaken in order to establish the localization of ARF1-regulated PLD activity in HL60 cells. Here we report that the ARF1-regulated PLD activity is present at membrane compartments enriched in Golgi/endoplasmic reticulum/granules (endomembranes) and at the plasma membrane. In addition, a substantial proportion of the ARF1-regulated PLD activity was also identified in the cytosol. In formyl-Met-Leu-Phe (FMLP)-stimulated cells, PLD activation was observed in both endomembranes and the plasma membrane.

MATERIALS AND METHODS

Materials

Culture media (RPMI and Medium 199) and supplements were purchased from Flow Laboratories. Foetal calf serum was from Imperial Laboratories. [2-^{14}C]Acetic acid (sodium salt), *myo*-[2-^{3}H]inositol, dipalmitoyl phosphatidyl[*methyl*-^{3}H]choline and [γ-^{32}P]ATP were obtained from Amersham. GTP[S] and ATP were purchased from Boehringer-Mannheim. Phosphatidylethanolamine (PE) and PIP_2 were obtained from Sigma, and PC was from Avanti. Recombinant ARF1 (rARF1) was purified from transfected *Escherichia coli* as described previously [5]. Rabbit polyclonal antibody to phosphatidylinositol transfer protein (PI-TP) was prepared as described previously [32]. 23C is a rat monoclonal anti-β'-COP antibody, where β'-COP is β' coat protein [33,34]. ID9 is a mouse monoclonal antibody to ARF1 and was generously donated by Dr. R. A. Kahn (National Cancer Institute, Bethesda, MD, U.S.A.). Rabbit polyclonal antibodies to G-protein common β-subunit and $G_i\alpha2$ were kindly provided by Dr. G. Milligan (University of Glasgow, Glasgow, U.K.). Anti-$G_i\alpha3$ antibody (rabbit polyclonal) was obtained from Calbiochem, and anti-RhoA antibody (mouse monoclonal) was from Santa Cruz Biotechnology Inc.

Production of monoclonal antibodies to ARF1

rARF1 was expressed in *E. coli* and purified [5]. Rats were immunized with rARF1 and hybridoma cell lines were isolated and cloned through two rounds of cell dilution and screening according to a standard schedule [35]. Six rat anti-ARF1 monoclonal antibodies were obtained: FRA/1/10a, 87, 23, 31b, 780w and 88b. Monoclonal antibodies 23 and 780w are IgG1 isotypes, 31b and 88b are IgG2a isotypes, 10a is IgG2b isotype and 87 is IgM isotype.

Growth and labelling of HL60 cells

HL60 cells were routinely maintained in suspension culture in RPMI 1640 media supplemented with 12.5% (v/v) foetal calf serum. HL60 cells were differentiated with dibutyryl-cAMP (300 μM) for 48 h when FMLP was used as a stimulus [36]. Cells labelled with [^{14}C]acetate were grown in the presence of 0.5 μCi/ml of the isotope through four growth cycles to ensure labelling close to equilibrium. Labelling with [^{3}H]inositol was as previously described [37].

Sucrose-density-gradient fractionation

Approx. (2–3) $\times 10^8$ HL60 cells were used for each gradient. Cells were washed twice with buffer A, comprising 137 mM NaCl, 2.7 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 20 mM Hepes, pH 7.2. After treatment on ice with 2 mM di-isopropyl-fluorophosphate for 10 min, cells were pelleted and washed once with ice-cold Harms buffer (250 mM sucrose, 10 mM triethanolamine, 10 mM acetic acid, 1 mM EDTA, pH 7.45). The cells were resuspended into 2–3 ml of the same buffer and a cocktail of protease inhibitors was added to give final concentrations of chymostatin, pepstatin and antipain of 5 μg/ml, leupeptin at 10 μg/ml and PMSF at 100 μM. After incubation on ice for 10 min, the cells were broken in a stainless steel ball-bearing homogenizer (H & Y Enterprise, Redwood City, CA, U.S.A.). The post-nuclear supernatant was fractionated on a continuous sucrose gradient of 10.4–40% (w/w), with a 1 ml 65% (w/w) cushion, exactly as described by Lewis et al. [38] except that 17 fractions of 1 ml were collected.

Assay of fractions for rARF1-dependent PLD activity utilizing endogenous substrate

Reconstitution of rARF1-dependent PLD activity was assessed by measuring the formation of [^{14}C]phosphatidylethanol ([^{14}C]PEt). Briefly, an aliquot (150 μl) of each fraction was incubated in the presence of 10 μM GTP[S], 2 mM $MgCl_2$, 1 mM MgATP, 1% ethanol, Ca^{2+} buffered with 3 mM EGTA (pCa 5) and either rARF1 (final concentration 40 μM) or an equivalent volume of Pipes buffer (137 mM NaCl, 2.7 mM KCl, 20 mM Pipes, 5.6 mM glucose, 0.1 mg/ml albumin, pH 6.8) in a final volume of 180 μl. Control incubations contained neither GTP[S] nor rARF1. After 60 min at 37 °C the assays were quenched by the addition of 5 vol. of chloroform/methanol (1:1, v/v). Following phase separation, the lower lipid-containing phase was collected, spiked with a mixture of standard lipids, dried under vacuum and redissolved in 50 μl of chloroform. Lipids were separated by TLC on Merck silica gel 60 TLC plates in a solvent system of chloroform/methanol/acetic acid/water (75:45:3:0.4, by vol.) and subsequently visualized with iodine vapour. R_F values were: PC, 0.073; phosphatidylserine, 0.145; phosphatidylinositol (PI), 0.19; PE, 0.455; PA, 0.53; PEt, 0.75; neutral lipids, 1.00. Each phospholipid spot was excised and the silica put into scintillation vials. The lipids were extracted with 0.2 ml of methanol and counted for radioactivity after addition of 3 ml of scintillation fluid (Canberra Packard). For an exam-

ination of the MgATP-dependence of rARF-stimulated PLD activity in separate membrane compartments, fractions coinciding with appropriate markers were pooled (endomembranes, fractions 2 and 3; plasma membrane, fractions 6 and 7). The pooled fractions were assayed exactly as above, except that MgATP was omitted from the assay mix. MgATP and neomycin were each included at 1 mM final concentration as indicated.

Localization of PEt formation in FMLP-stimulated cells

[^{14}C]Acetate-labelled differentiated HL60 cells were washed twice and resuspended in buffer A containing 5.6 mM glucose and 1 mg/ml BSA. Cytochalasin B and ethanol were added to the cells at final concentrations of 5 μM and 1 % (v/v) respectively. Cells were stimulated with FMLP (1 μM final) for 5 min at 37 °C. Control cells were incubated in the absence of the agonist. Stimulation was terminated by the addition of 10 vol. of ice-cold buffer A, and the cells were pelleted and processed for fractionation as described above. After fractionation, the lipids from each fraction (400 μl) were extracted as previously described [39] and PEt was analysed by TLC as described above.

Determination of subcellular markers

The distribution of subcellular markers was assessed on unlabelled gradients. Arylsulphatase (lysosomal marker) was assayed as described [40]. Briefly, the diluted fraction (50 μl) was incubated with an equal volume of assay mixture giving final concentrations of 0.1 M acetate buffer, pH 5.6, 3 mM lead acetate (freshly prepared), 0.1 % (v/v) Triton X-100 and 5 mM 4-methylumbelliferyl sulphate. The reaction was carried out at 37 °C for 30 min, quenched with 0.2 M Tris, pH 10.2, and the resulting fluorescence measured with a Titertek Fluoroscan II plate reader. β-Hexosaminidase was assayed in the presence of 0.1 % Triton X-100 using 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide [41]. The distribution of trans-Golgi membranes was determined by assaying for galactosyltransferase activity [42]. NADPH–cytochrome *c* reductase was assayed as a marker for the endoplasmic reticulum [43]. Human leucocyte antigen (HLA) class I (plasma membrane marker) was localized by ELISA as described [44]. For data presentation, the sum of enzyme activities in all fractions was taken as 100 %. Enzyme recoveries of β-hexosaminidase and arylsulphatase were in the range 120–130 %.

PI 4-kinase activity utilizing exogenous substrate was monitored in 50 μl portions of each fraction using 500 μM PI as substrate in a final assay volume of 100 μl; 1 % Triton X-100, 100 μM ATP, 20 mM $MgCl_2$ and 5 μCi of [γ-^{32}P]ATP were also included. Phosphatidylinositol 4-phosphate (PI4P) 5-kinase activity was assayed using exogenous substrate as described previously [45]. In brief, a 50 μl portion of each fraction was assayed using 40 μM PI4P as substrate in a final volume of 100 μl. The assay included 50 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EGTA, 50 μM ATP, 15 mM $MgCl_2$, 1 % Triton X-100, 400 μg/ml BSA and 5 μCi of [γ-^{32}P]ATP. Examination of kinase activity utilizing endogenous substrate was assayed as for PI4P 5-kinase, except that incubations were carried out in the absence of added phospholipids and Triton X-100. In all cases activities were assayed at 30 °C for 8 min and quenched with 750 μl of chloroform/methanol/conc. HCl (40:80:1, by vol.). After the further addition of 250 μl of chloroform and 250 μl of 0.1 M HCl/5 mM EDTA/0.5 M NaCl, each sample was vortexed vigorously and centrifuged to separate the chloroform phase from the aqueous phase. The organic phase was washed twice with 600 μl of a synthetic upper phase [chloroform/methanol/(0.1 M HCl, 5 mM EDTA, 0.5 M NaCl), 3:48:47, by vol.]. The chloroform phase was dried down and the lipids were resuspended in 50 μl of chloroform and loaded on to oxalate-treated TLC plates. The lipids were separated using chloroform/methanol/acetone/acetic acid/water (40:13:15:12:8, by vol.). The phosphorylated lipids were located by autoradiography and the lipids were quantified as described above.

Subcellular distribution of polyphosphoinositides

[^{3}H]Inositol-labelled HL60 cells were fractionated on sucrose gradients as above. A 180 μl portion of each fraction was incubated with 1 mM MgATP, 2 mM $MgCl_2$ and Ca^{2+} at pCa 7 in a final assay volume of 200 μl. After 20 min at 37 °C, the assays were quenched, the lipids were extracted with 900 μl of chloroform/methanol (1:1, v/v) and the phases were separated with 450 μl of 1 M HCl. Lipids were separated by TLC and quantified as described above.

Assay of rARF1-regulated PLD activity using exogenous substrate

Sucrose-density-gradient fractions were assayed either fresh, or thawed from storage at −20 °C, essentially as previously described [46]. Sucrose at the concentrations present in the fractions was not found to interfere with this assay. Briefly, fractions (10 μl) were assayed in duplicate, with the addition of either GTP[S] alone or both GTP[S] and rARF1 as indicated. Assay cocktails were as described [46]. GTP[S] was used at 30 μM, rARF1 at 20 μM and NaCl at 440 mM final concentrations. Substrate was prepared from PE, PIP_2 and PC in the molar ratio 42:4:1. Labelled PC ([*methyl*-^{3}H]choline) was added to give approx. 100000 d.p.m. per assay tube. Reactions were incubated for 1 h at 37 °C, followed by quenching; radiolabelled choline released was monitored as described [46]. Data are presented as pmol of PC hydrolysed.

SDS/PAGE and immunoblotting

After SDS/PAGE [47], proteins were either visualized by Coomassie Blue staining or analysed by immunoblotting for marker enzymes. Western blotting on to poly(vinylidene difluoride) membranes was performed by established procedures. Detection was by an enhanced chemiluminescence technique (Amersham). Blots were probed with specific antisera against ARF, RhoA, PI-TP, $G_i\alpha3$, $G_i\alpha2$, the common β-subunit of heterotrimeric G-proteins and p102/β'-COP.

RESULTS

Subcellular localization of ARF1-regulated PLD activity using [^{14}C]acetate-labelled cells

To identify the subcellular localization of the ARF1-regulated PLD activity, a cell fractionation procedure based on linear sucrose gradients was used [38]. The cellular lipids were prelabelled to equilibrium by growing HL60 cells in the presence of [^{14}C]acetate, and PLD activity was monitored in individual fractions by measuring the production of radiolabelled PEt (Figure 1A). GTP[S] alone stimulated PLD activity in fraction 2, which most probably utilizes the endogenous ARF proteins present in this fraction (see Figure 4b). Addition of both GTP[S] and rARF1 revealed a major peak of PLD activity in fractions 2–3 and a second smaller peak in fractions 7–8.

The magnitude of PEt production ultimately depends upon the amount of the labelled substrate present in these fractions.

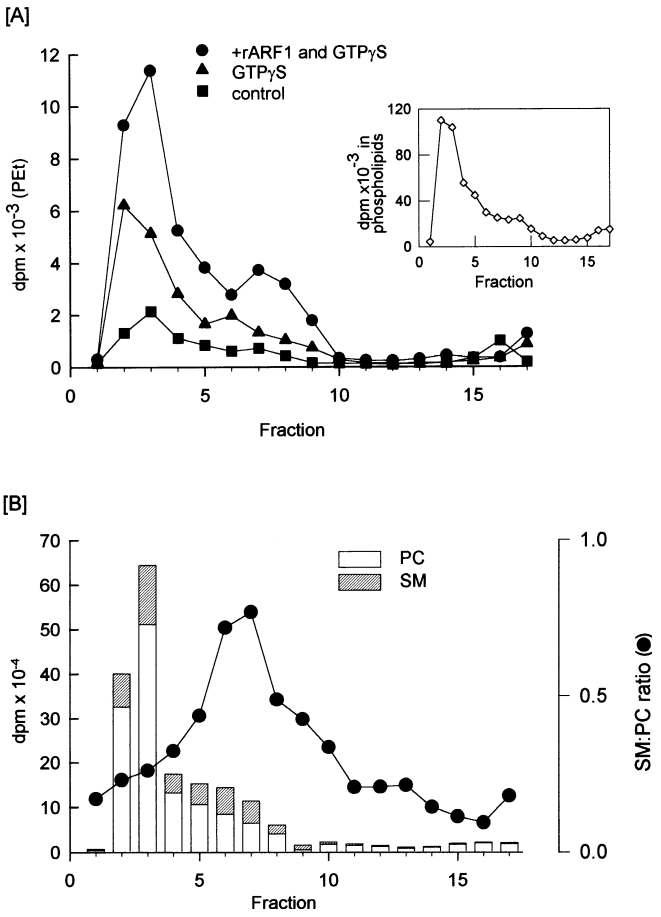

Figure 1 Subcellular localization of rARF1-stimulated PLD activity using labelled endogenous lipids as substrate

(**A**) HL60 cells prelabelled with [^{14}C]acetate were disrupted and fractionated on a sucrose gradient as described in the Materials and methods section. Fractions were collected from the bottom of the gradient. An aliquot (150 μl) of each fraction was incubated with 10 μM GTP[S] (GTPγS) in the presence or absence of 40 μM rARF1 as indicated, for 60 min. Ethanol (1%) was included in each incubation, and the production of PEt was quantified as described in the Materials and methods section. The inset illustrates the distribution of total labelled phospholipids in the gradient. (**B**) The lipids were extracted and analysed by TLC to quantify PC and sphingomyelin (SM). The ratio of sphingomyelin to PC is also plotted. The data shown in (**A**) and (**B**) are representative of three independent experiments.

The distribution of the total phospholipids is shown as an inset in Figure 1(A). PC has been identified previously as the preferred substrate for PLD in many cell types [21,48,49], including HL60 cells [50], and its distribution is illustrated in Figure 1(B). Analysis of the phospholipid composition of each subcellular fraction revealed significant differences. In fraction 7, sphingomyelin and phosphatidylserine were highly enriched, whereas PC and PI were relatively depleted (results not shown). The sphingomyelin/PC ratio was found to approach 1.0 in fraction 7 (Figure 1B), compared with a value of 0.2 found in whole cells [39]. This phospholipid composition is characteristic of plasma membranes, as is the ratio of sphingomyelin to PC [39,51].

Analysis of gradient fractions using exogenous PC as a substrate for PLD

The subcellular fractions were also assayed for rARF1-stimulated PLD activity using vesicles containing PIP_2/PE/PC

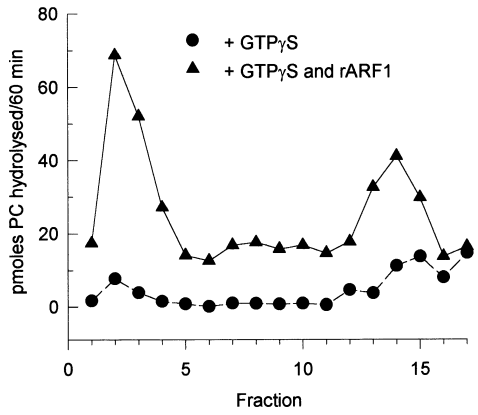

Figure 2 Subcellular localization of rARF1-stimulated PLD activity using exogenous [^{3}H]PC as substrate

HL60 cells were disrupted and fractionated as described in the Materials and methods section. rARF1-stimulated PLD activity was assessed in each fraction by measuring the release of free [^{3}H]choline from exogenously added phospholipid vesicles, as described in the Materials and methods section. Data from a representative experiment (one of six) are shown. GTPγS = GTP[S].

([^{3}H]dipalmitoyl-PC) [6] (Figure 2). GTP[S] alone gave a small stimulation of PLD activity in fraction 2 and in fractions 14–15. This stimulation by GTP[S] most probaby utilizes endogenous ARF/Rho proteins, since these fractions were shown to contain them (see Figures 4b, 4c and 4i). When the fractions were analysed with rARF1 alone, no activity was seen (results not shown). Addition of both GTP[S] and rARF1 increased PLD activity in all fractions, with two major peaks of activity. One peak was in fractions 2–3 and the second was in fractions 13–15 (Figure 2). A third region of activity was also observed in fractions 7–10. In three out of six experiments this activity was observed as a distinct peak.

Characterization of the sucrose gradient

The sucrose gradient was analysed by assaying for marker enzyme activities (Figure 3) and for specific proteins by Western blotting (Figure 4). Fractions 2–3 were enriched in markers for the Golgi complex (galactosyltransferase) (Figure 3a) and the endoplasmic reticulum (NADPH–cytochrome *c* reductase) (Figure 3b) as well as lysosomal granules (arylsulphatase and β-hexosaminidase) (Figures 3c and 3d). The sucrose concentrations of the labelled and unlabelled gradients from this run are also presented here (Figure 3f).

The plasma membrane was found to be highly enriched in fractions 6–9, as assessed by the distribution of HLA class I across the gradient (Figure 3e). This localization for the plasma membrane is supported by the characteristic phospholipid composition of these fractions, as discussed above. The contamination of the plasma membrane fractions with endomembranes was minimal, judging by the low levels of marker enzymes for Golgi, endoplasmic reticulum and lysosomal granules.

SDS/PAGE analysis of the subcellular fractions showed marked differences in the Coomassie Blue-stained protein pattern across the gradient (Figure 4a). The gradient can be visually subdivided into distinct zones (e.g. fractions 1–4, 5–6, 7–9 and 13–17). From the marker analysis, fractions 1–4 were enriched in endomembranes, fractions 6–9 in plasma membrane and fractions 12–16 in soluble proteins. The same fractions shown in Figure 4(a) were Western-blotted for ARF (antibody ID9; Figure

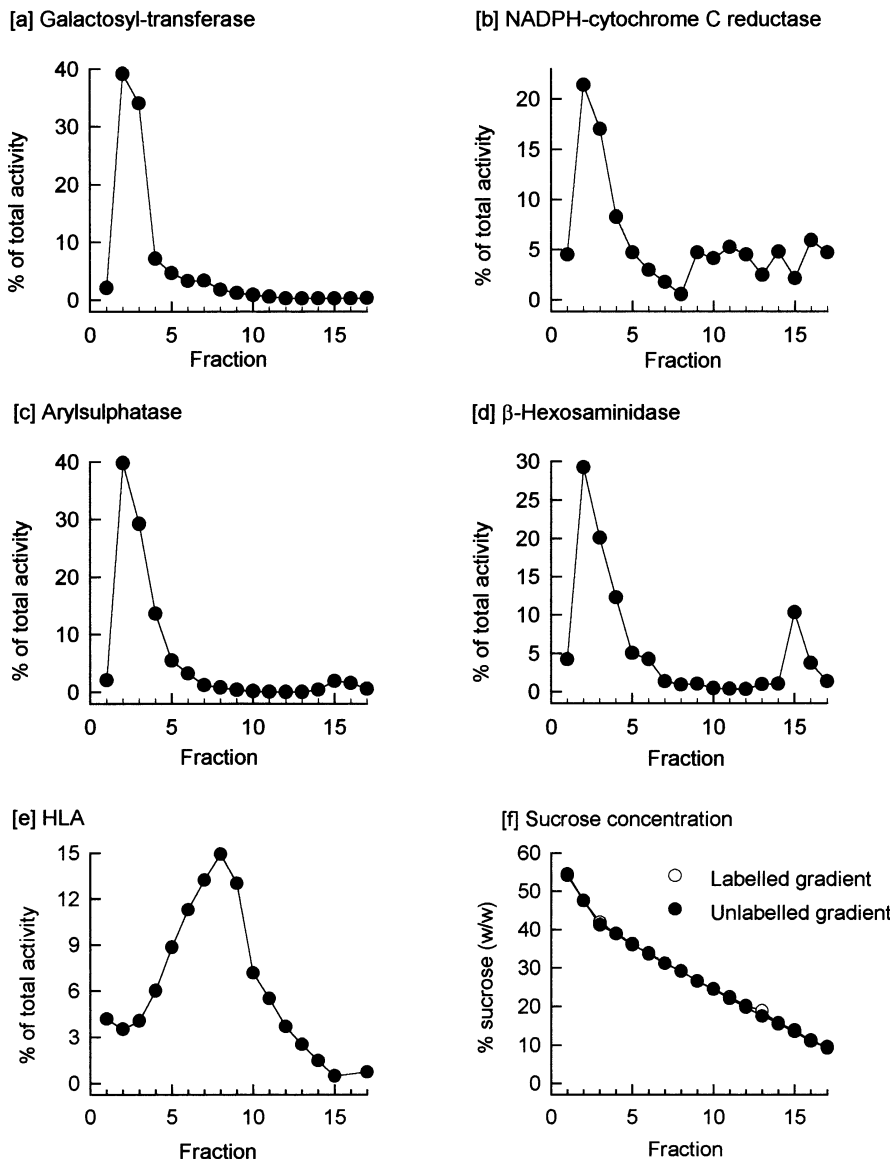

Figure 3 Distribution of subcellular markers in fractionated HL60 cells

HL60 cells were homogenized and fractionated on a sucrose gradient as described in the Materials and methods section. Fractions were collected from the bottom of the gradient. Marker analysis of each fraction was performed as described in the Materials and methods section. (**a**) Galactosyltransferase (marker for Golgi complex), (**b**) NADPH–cytochrome *c* reductase (endoplasmic reticulum marker), (**c**) arylsulphatase (lysosomal granule marker), (**d**) β-hexosaminidase (lysosomal granule marker), (**e**) HLA (plasma membrane marker), (**f**) sucrose concentration, as determined by refractometry. For each marker the sum of activities in all fractions was taken as 100%. These results are representative of at least four separate fractionations.

4b), PI-TP (Figure 4d), p102 (β'-COP) (Figure 4e) and $G_i\alpha3$ (Figure 4f). Two separate monoclonal antibodies against ARF were used; one antibody (ID9) has been widely used in previous studies (Figure 4b) and the other was a mixture of rat monoclonal antibodies that we prepared (Figure 4c). Both sets of antibodies gave similar results. [The data in Figure 4(c) were obtained from a separate fractionation.] Anti-ARF antibodies identified two regions; fractions 1–4 (endomembranes) showed a relatively weak signal, while fractions 15–17 (cytosol) contained the bulk of the ARF proteins. Figure 4(e) illustrates the localization of β'-COP (p102) in fractions 11–13. β'-COP is a subunit of the large macromolecular Golgi coatomer complex of 500–600 kDa, and consequently enters the gradient [33]. Co-localization of ARF and β'-COP was not observed. RhoA was localized mainly in the cytosol, with immunoreactivity also detected at both the plasma membrane and endomembranes (Figure 4i).

Previous studies have shown that PI-TP, a 35 kDa protein, is present exclusively in the cytosol in HL60 cells [32], and here it was found in fractions 15–16 (Figure 4d). Antibodies to $G_i\alpha3$, $G_i\alpha2$ and β-subunit (common) of G-proteins identified two distinct membrane regions (fractions 2–4 and fractions 7–9) for these proteins (Figures 4f, 4g and 4h respectively). As described above, fractions 2–3 are enriched in endomembranes and fractions 7–9 represent the plasma membrane. This localization of G-protein subunits is in agreement with previous studies where G-proteins, including $G_i\alpha3$, have been found not only at the plasma membrane but also at the Golgi complex [52,53]. Some β-subunits were also identified in the cytosolic region

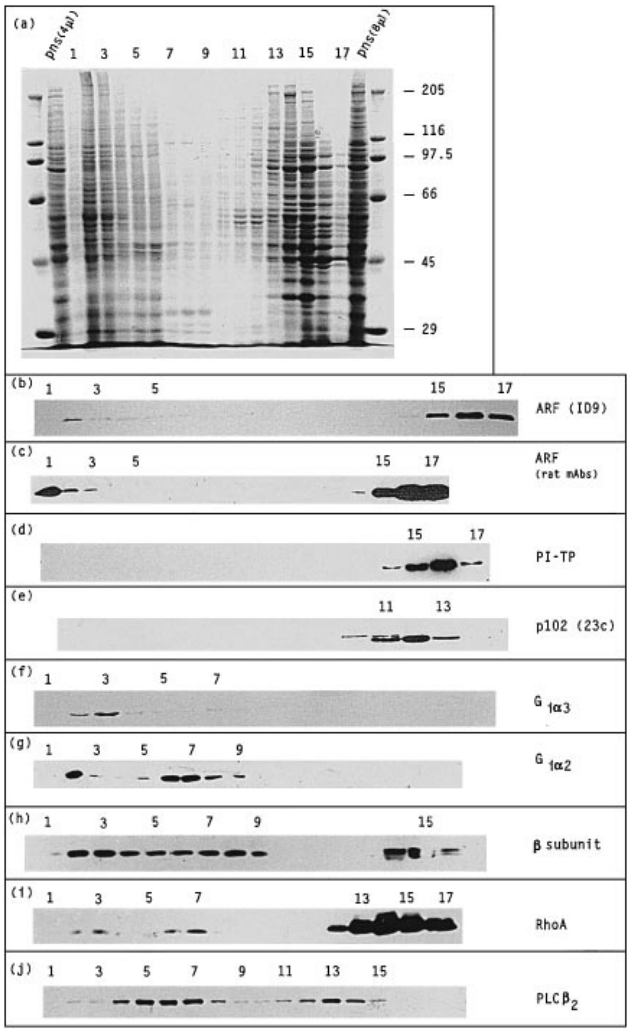

Figure 4 Distribution of proteins and subcellular marker antigens in the sucrose gradient

HL60 cells were homogenized and fractionated as described in the Materials and methods section. Proteins in each fraction were analysed by SDS/PAGE and either stained with Coomassie Blue (**a**) or Western blotted with specific antibodies to: (**b**) ARF (ID9), (**c**) ARF (rat monoclonals), (**d**) PI-TP, (**e**) β'-COP/p102, (**f**) $G_i\alpha3$, (**g**) $G_i\alpha2$, (h) common G-protein β-subunit, (**i**) RhoA and (**j**) phospholipase $C\beta_2$. Numbers above the lanes are fraction numbers; pns is post-nuclear supernatant. In (**a**), molecular masses (kDa) of marker proteins are indicated on the right. These results are representative of at least four separate fractionations.

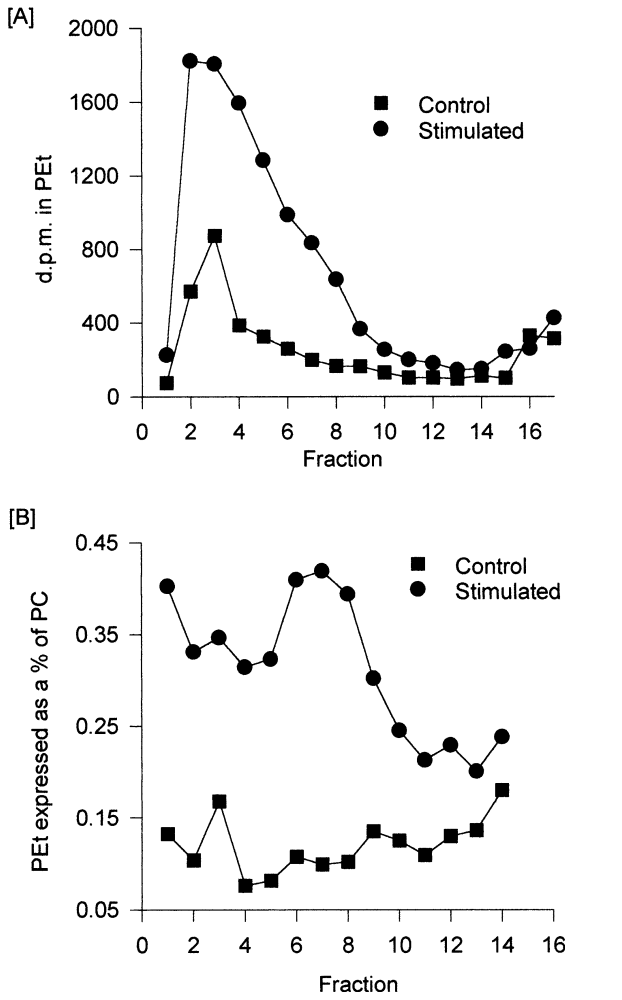

Figure 5 Subcellular distribution of FMLP-stimulated PLD activity in intact HL60 cells

HL60 cells (differentiated), prelabelled with [^{14}C]acetate, were stimulated with 1 μM FMLP for 5 min as indicated, in the presence of 1% ethanol. After quenching the reaction, the cells were disrupted and fractionated as described in the Materials and methods section. Each fraction was analysed for HLA (plasma membrane marker) and [^{14}C]PEt as described in the Materials and methods section. As in undifferentiated HL60 cells, HLA peaked in fractions 6–8 for both control and stimulated cells (results not shown). All data shown are from a representative experiment which was carried out twice with identical results. (**A**) Distribution of the absolute amount of [^{14}C]PEt in the subcellular fractions in control and stimulated cells; (**B**) PEt levels expressed as a percentage of the PC in each fraction.

(Figure 4h). Phospholipase C-β_2 was found in fractions 4–7 as well as in the cytosolic region (Figure 4j). (HLA in this specific run was localized in fractions 6–8.) This localization is in agreement with the plasma membrane being the site for phospholipase C signalling in neutrophils [54], but additionally there is immunoreactivity in denser fractions which remains currently undefined.

Subcellular localization of FMLP-stimulated PLD activity

It is clear from the results described above that ARF-stimulated PLD activity is present in both endomembranes and the plasma membrane. To examine which of the activities is responsible for PEt production in intact cells, we analysed the distribution of PEt in the subcellular fractions from control and FMLP-stimulated cells. Differentiated HL60 cells prelabelled with [^{14}C]acetate were stimulated with FMLP in the presence of ethanol and fractionated after disruption, and PEt levels were determined in each fraction. The absolute radioactivity (d.p.m.) in PEt was greatest in fractions 2–3 (Figure 5A), with a distinct shoulder seen in fractions 6–8. The shoulder in fractions 6–8 is observed as a distinct peak when the data are expressed as a function of the amount of PC present in the individual fractions (Figure 5B).

MgATP requirement for rARF1-stimulated PLD activity

It has been demonstrated previously that rARF1-stimulated PLD activity is enhanced by MgATP [3,55,56]. This MgATP requirement may represent the availability of PIP_2 produced by sequential phosphorylation of PI by PI 4-kinase and PI4P 5-

Table 1 Comparison of MgATP-dependence of ARF-regulated PLD activity at plasma membrane and endomembranes: inhibition by neomycin

HL60 cells prelabelled with [^{14}C]acetate were disrupted and the homogenate was fractionated on a sucrose gradient as described in the Materials and methods section. Fractions enriched in endomembranes and plasma membranes were pooled and the MgATP-dependence of the rARF1-regulated PLD activity was assessed. An aliquot (100 μl) of each pooled fraction was incubated with 10 μM GTP[S] and 40 μM rARF1 in the presence or absence of MgATP (1 mM) and neomycin (1 mM) as indicated. Values in parentheses are PEt production as a percentage of the control.

Additions	[^{14}C]PEt formed (d.p.m.)	
	Control	rARF1 + GTP[S]
Endomembranes		
None	651 (100%)	1586 (244%)
MgATP	1962 (301%)	12220 (1877%)
MgATP + neomycin	774 (119%)	1045 (160%)
Plasma membranes		
None	177 (100%)	738 (417%)
MgATP	276 (156%)	1571 (888%)
MgATP + neomycin	177 (100%)	561 (317%)

kinase. PIP_2 has been shown to be an obligatory requirement for rARF-regulated PLD activity, not just *in vitro* [6] but also when analysed in membranes using endogenous substrate [56]. We therefore analysed the MgATP-dependence of the ARF1-regulated PLD activity in [^{14}C]acetate-labelled HL60 cells. The endomembrane-enriched fractions 2–3 were pooled, as were the plasma membrane-enriched fractions 6–7. The pooled fractions were incubated in the presence or absence of both GTP[S] and rARF1 (Table 1). In endomembranes, a low level of rARF1-stimulated PLD activity was observed, which was enhanced 8-fold by the presence of 1 mM MgATP. In contrast, a robust rARF1-dependent activity was found at the plasma membrane which was only increased 2-fold by MgATP (Table 1). Neomycin sequesters PIP_2 and has been recently found to be a potent inhibitor of rARF1-reconstituted activity in human neutrophils [56]. In the presence of MgATP, the rARF1-stimulated PLD activity associated with the endomembranes was inhibited by neomycin to a greater extent than the activity at the plasma membrane (Table 1).

Distribution of polyphosphoinositides in the subcellular fractions

Since the assay of ARF1-regulated PLD activity using exogenous or endogenous substrate has identified a role for PIP_2 as a cofactor, we examined the subcellular localization of this lipid. The distributions of PI 4-kinase and PI4P 5-kinase activities are shown in Figure 6. The fractions were analysed both using the endogenous lipids as substrates and also using exogenous lipid in the presence of 1% Triton X-100. PI 4-kinase was localized to fractions 2–3 and additionally to fractions 6–8, in agreement with previous reports that this enzyme is localized at the Golgi, lysosomes and plasma membranes [57] (Figure 6A). When assayed using endogenous substrate (Fig 6A, inset), PI4P was formed mainly in fraction 2, with some in fractions 5–7. PI4P 5-kinase activity was found to be primarily localized in fractions 6–9 when assayed using both exogenous and endogenous substrate (Figure 6B, inset), although a small amount of activity was consistently detected in fractions 2–3 (Figure 6B).

The distribution of [^{3}H]inositol-labelled lipids in HL60 cells was also examined after fractionation on the sucrose gradient. The fractions obtained from [^{3}H]inositol-labelled cells were

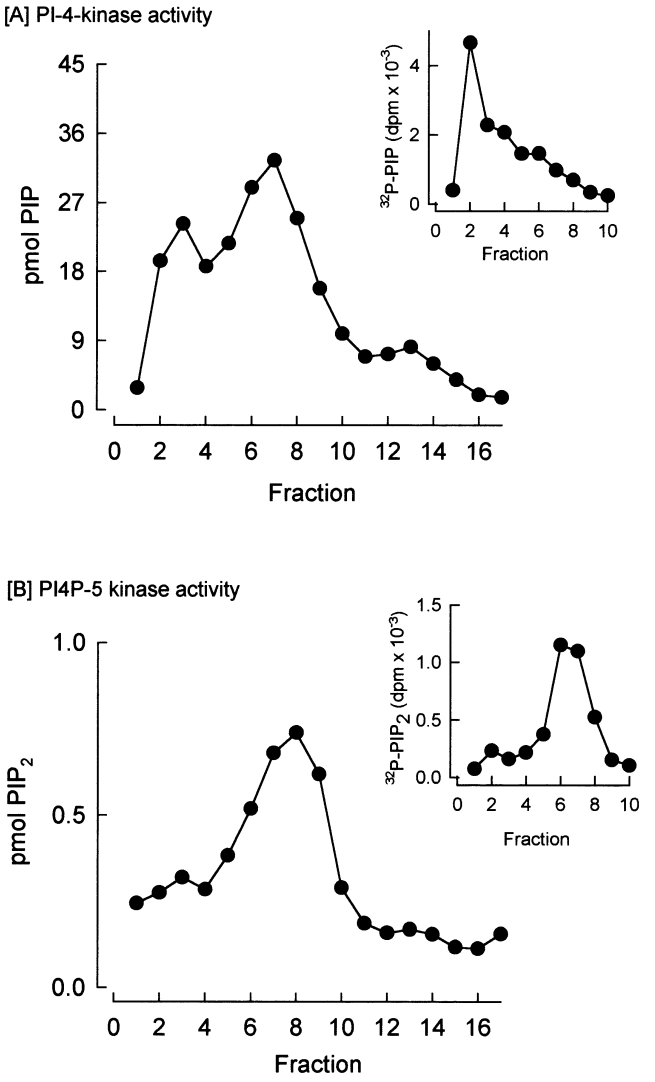

Figure 6 Distribution of PI 4-kinase and PI4P 5-kinase in the sucrose gradient

HL60 cells were homogenized and fractionated as described in the Materials and methods section. (**A**) Assay of PI 4-kinase activity. An aliquot (50 μl) of each fraction was incubated with 500 μM PI, 1% Triton X-100 and [γ-^{32}P]ATP, and the radioactivity incorporated into PI4P (PIP) was analysed as described in the Materials and methods section. The inset shows the distribution of kinase activity utilizing endogenous substrate. (**B**) Assay of PI4P 5-kinase activity. Fractions (50 μl) were incubated with 40 μM PI4P, 1% Triton X-100 and 5 μCi of [γ-^{32}P]ATP, and PIP_2 production was analysed as above. The inset shows the localization of enzyme when endogenous substrate was utilized. The data in (**A**) and (**B**) are representative of four separate experiments.

incubated with MgATP to ensure that the endogenous inositol lipids were phosphorylated to their maximal capacity by the endogenous lipid kinases. The distributions of PI and PI4P reflected the distributions of the total lipids in the individual fractions (compare inset to Figure 1A with Figures 7b and 7c). In contrast, the distribution of PIP_2 did not reflect the distribution of the total lipids but was highly enriched in fractions 6 and 7, the region identified as plasma membranes by marker enzyme analysis and its distinct phospholipid composition (Figure 7a). The endomembrane region has some PIP_2 associated with it, supporting the presence of some PI4P 5-kinase activity.

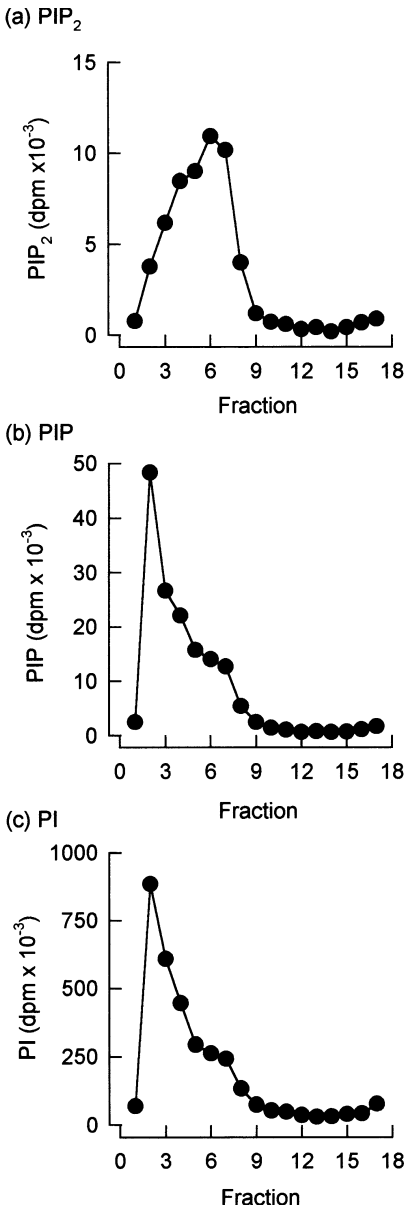

Figure 7 Distribution of inositol-containing phospholipids in fractionated [^{3}H]inositol-labelled HL60 cells

HL60 cells labelled with [^{3}H]inositol were disrupted and fractionated as described in the Materials and methods section. An aliquot (180 μl) of each fraction was incubated with 1 mM MgATP to maximally phosphorylate the endogenous inositol lipids. [^{3}H]Inositol-labelled lipids were quantified as described in the Materials and methods section. A representative (one of three) experiment is shown. PIP = PI4P.

DISCUSSION

HL60 cells and neutrophils are two of the most extensively studied cell types with respect to the regulation of PLD activity. Previous studies have established that the PLD activity in these cells is located in the membrane fraction and that its stimulation by guanine nucleotides is mediated by small GTP-binding proteins belonging to the ARF family [5,6,56]. The mammalian ARF family comprises five proteins belonging to three classes. Class I contains ARF1 and ARF3, which are 96% identical, class II contains ARF4 and class III contains ARF5 and ARF6. Members of all three classes are able to stimulate PLD activity [20]. Using subcellular fractionation, we demonstrate that rARF1-regulated PLD activity is localized not only in intracellular compartments but also at the plasma membrane and in the cytosol.

The cytosolic nature of the PLD activity was confirmed when a high-speed supernatant was prepared from HL60 cells and assayed for ARF1-regulated PLD activity. Since the cytosolic PLD activity migrated into the sucrose gradient to fractions 12–14, it can be inferred that the activity migrates as a macromolecule with an approximate size of 200–300 kDa [38]. This estimate is in line with a recent report where the cytosolic PLD activity from HL60 cells was analysed by gel filtration and was estimated to have a molecular mass of >150 kDa [16]. In addition, the PLD activity purified from pig lung has a molecular mass of 190 kDa on SDS/PAGE [21]. However, it is considerably larger than the recently cloned hPLD1 [22]. Smaller proteins such as ARF and Rho (21 kDa) and PI-TP (35 kDa) are well separated from the cytosolic PLD activity and migrate in fractions 15–17. In contrast, β'-COP, which is a part of a large macromolecular complex of 600 kDa, migrates in fractions 11–12.

Subcellular fractionation of HL60 cells on continuous sucrose gradients gave a clean separation of the plasma membrane from other intracellular compartments. The markers for the Golgi complex, endoplasmic reticulum and lysosomal granules co-sedimented at the bottom of the gradient. In addition, approx. 10–15% of the plasma membrane marker (Figure 3e) was detected in these dense fractions. Many attempts were made to further separate these membrane compartments using Nycodenz gradients, but this was unsuccessful in HL60 cells. [Although we have not succeeded in separating these compartments in HL60 cells, studies with human neutrophils indicate that the azurophilic (lysosomal) granules have no detectable rARF1-stimulated PLD activity.] Ktistakis et al. [31] have recently shown that a brefeldin A-sensitive ARF-activated PLD activity is localized at the Golgi complex but not in endoplasmic reticulum-enriched membranes.

The presence of rARF1-regulated PLD activity at the plasma membrane indicates that ARF proteins are functional not only at intracellular membranes but also at the plasma membrane. Recently, ARF6 when overexpressed was found to be localized at the plasma membrane, and has been suggested to be involved in endocytosis [58]. It is possible that ARF1 is able to replace ARF6 *in vitro*.

It has been reported that the inclusion of PIP_2 in PC/PE vesicles is crucial for the demonstration of ARF-stimulated PLD activity as well as RhoA-stimulated PLD activity [16] under *in vitro* assay conditions. In membranes or permeabilized cells where endogenous substrate is used, a requirement for PIP_2 is also indicated [56]. The majority of PIP_2 is reported to be localized at the plasma membrane [57,59,60]. To examine the presence of PIP_2 in endomembranes, where the major component of ARF-regulated PLD activity is found, the subcellular distributions of the inositol lipids and the associated kinases responsible for inositol lipid phosphorylation were analysed. PI4P and PI 4-kinase activity were present in intracellular compartments and the plasma membrane. As expected, PI4P 5-kinase activity was predominantly found at the plasma membrane whether activity was monitored using endogenous or exogenous substrate. However, a small but consistent amount of activity was also observed in endomembranes. The PI4P 5-kinase data were supported by an examination of the distribution of PIP_2 in the gradient. These results are consistent with previous reports that PIP_2 synthesis occurs in a microsomal fraction of CHO cells distinct from the plasma membrane [60]. It appears that there is

sufficient PIP_2 present at endomembranes to fulfil the requirements for rARF1-regulated PLD activity. The presence of PIP_2 in endomembranes is also supported by the identification of a PIP_2 5-phosphatase at the Golgi complex [61,62]. PIP_2 has been shown to catalyse the release of GDP from rARF1 *in vitro*, as well as stabilizing the apo-form of ARF1 [63]. In addition, PIP_2 can stimulate ARF-GTPase-activating protein activity [64]. Thus PIP_2 clearly plays a complex role in ARF function.

To further examine this PIP_2-dependence, MgATP and neomycin were used to modulate the availability of PIP_2. In the presence of MgATP, there is an increase in the cellular content of PIP_2 [65], conditions which potentiated rARF1-stimulated PLD activity in both endomembranes and plasma membranes. Endomembranes displayed a greater sensitivity to MgATP and neomycin, indicating that there is only a limited pool of PIP_2 in intracellular organelles relative to the plasma membrane.

Many proteins, including those possessing pleckstrin homology domains (such as β-adrenergic receptor kinase, pleckstrin and Ras-GTPase-activating protein) and cytoskeletal proteins (such as gelsolin and profilin), specifically bind PIP_2 [66,67]. If PIP_2 is responsible in part for the association of PLD with membranes *in vivo*, the observation that some of the ARF-regulated PLD activity was found in the cytosol could be artefactual. The homogenization buffer does not contain MgATP, and therefore PIP_2 levels would become depleted during preparation of the high-speed supernatant and during fractionation of the homogenate on sucrose gradients.

The localization of $G_i\alpha 3$ and $\beta\gamma$-subunits of G-proteins at intracellular organelles observed here supports their proposed roles in membrane trafficking events [68]. Heterotrimeric G-proteins may participate in the regulation of vesicular trafficking by interacting with ARF directly [69,70].

In summary, we demonstrate that ARF-regulated PLD activity is present not only at endomembranes but also at the plasma membrane. Both of these activities are stimulated upon receptor occupation by FMLP, as judged by the formation of PEt in these membrane compartments. A previous study had reported that PEt was formed exclusively at the plasma membrane upon stimulation with FMLP [71]. However, that study relied on the use of lyso-platelet-activating factor to prelabel the cells, and thus it is not clear whether the label had been fully distributed to the intracellular compartments. The labelling procedure used in the present study was designed to label all the phospholipids to near equilibrium. Our study clearly indicates that FMLP stimulates a substantial degree of PEt production in intracellular compartments. The degree of PEt formed in the different membrane compartments upon receptor stimulation reflects the distribution of the PLD activity. The intracellular localization of the ARF-regulated PLD activity is indicative of it playing a prominent role in vesicular transport. The identification of ARF-regulated PLD activity at the plasma membranes poses interesting questions concerning PA metabolism. The plasma membrane is also the site for G-protein-stimulated phospholipase C activity; here the immediate product of lipid hydrolysis is diacylglycerol, which is phosphorylated to PA within seconds. The product of PLD activity is PA, and this is metabolized to diacylglycerol by a plasma-membrane-localized phosphatidate phosphohydrolase. The production of these lipid metabolites (diacylglycerol and PA) by phospholipases C and D respectively must be segregated at the plasma membrane to avoid futile interconversions.

We thank the Medical Research Council, the Wellcome Trust and the Cancer Research Campaign for financial support. We thank Dr. R. A. Kahn for anti-ARF antibodies and Dr. G. Milligan for antibodies to the β-subunit of G-proteins and to $G_i\alpha 2$. We thank Jackie Cordell (ICR Hybridoma Unit) for production of rat anti-ARF1 monoclonal antibodies.

REFERENCES

1 Olson, S. C., Bowman, E. P. and Lambeth, J. D. (1991) J. Biol. Chem. **266**, 17236–17242
2 Dubyak, G. R., Schomisch, S. J., Kusner, D. J. and Xie, M. (1993) Biochem. J. **292**, 121–128
3 Geny, B. and Cockcroft, S. (1992) Biochem. J. **284**, 531–538
4 Anthes, J. C., Wang, P., Siegel, M. I., Egan, R. W. and Billah, M. M. (1991) Biochem. Biophys. Res. Commun. **175**, 236–243
5 Cockcroft, S., Thomas, G. M. H., Fensome, A., Geny, B., Cunningham, E., Gout, I., Hiles, I., Totty, N. F., Troung, O. and Hsuan, J. J. (1994) Science **263**, 523–526
6 Brown, H. A., Gutowski, S., Moomaw, C. R., Slaughter, C. and Sternweis, P. C. (1993) Cell **75**, 1137–1144
7 Serafini, T., Orci, L., Amherdt, M., Brunner, M., Kahn, R. A. and Rothman, J. E. (1991) Cell **67**, 239–253
8 Rothman, J. E. and Orci, L. (1992) Nature (London) **355**, 409–415
9 Zhang, C., Rosenwald, A. G., Willingham, M. C., Skuntz, S., Clark, J. and Kahn, R. A. (1994) J. Cell Biol. **124**, 289–300
10 Donaldson, J. G. and Klausner, R. D. (1994) Curr. Opin. Cell Biol. **6**, 527–532
11 Stearns, T., Willingham, M. C., Botstein, D. and Kahn, R. A. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 1238–1242
12 De Matteis, M. A., Santini, G., Kahn, R. A., Di Tullio, G. and Luini, A. (1993) Nature (London) **364**, 818–821
13 Cukierman, E., Huber, I., Rotman, M. and Cassel, D. (1995) Science **270**, 1999–2002
14 Fensome, A., Cunningham, E., Prosser, S., Tan, S. K., Swigart, P., Thomas, G., Hsuan, J. and Cockcroft, S. (1996) Curr. Biol. **6**, 730–738
15 Bowman, E. P., Uhlinger, D. J. and Lambeth, J. D. (1993) J. Biol. Chem. **268**, 21509–21512
16 Siddiqi, A. R., Smith, J. L., Ross, A. H., Qiu, R., Symons, M. and Exton, J. H. (1995) J. Biol. Chem. **270**, 8466–8473
17 Malcolm, K. C., Ross, A. H., Qiu, R., Symons, M. and Exton, J. H. (1994) J. Biol. Chem. **269**, 25951–25954
18 Kuribara, H., Tago, K., Yokozeki, T., Sasaki, T., Takai, Y., Morii, N., Narumiya, S., Katada, T. and Kanaho, Y. (1995) J. Biol. Chem. **270**, 25667–25671
19 Singer, W. D., Brown, H. A., Bokoch, G. M. and Sternweis, P. C. (1995) J. Biol. Chem. **270**, 14944–14950
20 Massenburg, D., Han, J., Liyanage, M., Patton, W. A., Rhee, S. G., Moss, J. and Vaughan, M. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 11718–11722
21 Okamura, S. and Yamashita, S. (1994) J. Biol. Chem. **269**, 31207–31213
22 Hammond, S. M., Altshuller, Y. M., Sung, T., Rudge, S. A., Rose, K., Engebrecht, J., Morris, A. J. and Frohman, M. A. (1995) J. Biol. Chem. **270**, 29640–29643
23 Wang, P., Anthes, J. C., Siegel, M. I., Egan, R. W. and Billah, M. M. (1991) J. Biol. Chem. **266**, 14877–14880
24 Cockcroft, S. (1992) Biochim. Biophys. Acta **1113**, 135–160
25 Billah, M. M. (1993) Curr. Opin. Immunol. **5**, 114–123
26 Exton, J. H. (1994) Biochim. Biophys. Acta **1212**, 26–42
27 Schmidt, M., Rumenapp, U., Bienek, C., Keller, J., von Eichel-Streiber, C. and Jakobs, K. H. (1996) J. Biol. Chem. **271**, 2422–2426
28 Kumada, T., Miyata, H. and Nozawa, Y. (1993) Biochem. Biophys. Res. Commun. **191**, 1363–1368
29 Nickel, W., Huber, L. A., Kahn, R. A., Kipper, N., Barthel, A., Fasshauer, D. and Söling, H. (1994) J. Cell Biol. **125**, 721–732
30 Stamnes, M. A. and Rothman, J. E. (1993) Cell **73**, 999–1005
31 Ktistakis, N. T., Brown, A., Sternweis, P. C. and Roth, M. G. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 4952–4956
32 Cunningham, E., Thomas, G. M. H., Ball, A., Hiles, I. and Cockcroft, S. (1995) Curr. Biol. **5**, 775–783
33 Harrison-Lavoie, K. J., Lewis, V. A., Hynes, G. M., Collison, K. S., Nutland, E. and Willison, K. R. (1993) EMBO J. **12**, 2847–2853
34 Willison, K., Lewis, V., Zuckerman, K. S., Cordell, J., Dean, C., Miller, K., Lyon, M. F. and Marsh, M. (1989) Cell **57**, 621–632
35 Dean, C. (1992) in Immunochemical Protocols (Manson, M., ed.), pp. 43–63, Humana Press Inc., New York
36 Nielson, C. P., Stutchfield, J. and Cockcroft, S. (1991) Biochim. Biophys. Acta **1095**, 83–89
37 Stutchfield, J. and Cockcroft, S. (1988) Biochem. J. **250**, 375–382
38 Lewis, V. A., Hynes, G. M., Zheng, D., Saibil, H. and Willison, K. (1992) Nature (London) **358**, 249–252
39 Whatmore, J., Quinn, P. and Allan, D. (1993) Biochim. Biophys. Acta **1152**, 300–306
40 Chang, P. L., Rosa, N. E. and Davidson, R. G. (1981) Anal. Biochem. **117**, 382–389

41 Stutchfield, J. and Cockcroft, S. (1993) Biochem. J. **293**, 649–655
42 Kim, Y. S., Perdomo, J. and Nordberg, J. (1971) J. Biol. Chem. **246**, 5466–5476
43 Evans, W. H. (1980) in Laboratory Techniques in Biochemistry and Molecular Biology (Work, T. S. and Work, E., eds.), pp. 1–266, Elsevier, New York
44 Bjerrum, O. W. and Borregaard, N. (1990) Scand. J. Immunol. **31**, 305–313
45 Moritz, A., de Graan, P. N. E., Gispen, W. H. and Wirtz, K. W. A. (1992) J. Biol. Chem. **267**, 7207–7210
46 Vinggaard, A. M., Jensen, T., Morgan, C. P., Cockcroft, S. and Hansen, H. S. (1996) Biochem. J. **319**, 861–864
47 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
48 Mohn, H., Chalifa, V. and Liscovitch, M. (1992) J. Biol. Chem. **267**, 11131–11136
49 Horwitz, J. and Davis, L. L. (1993) Biochem. J. **295**, 793–798
50 Pai, J., Siegel, M. I., Egan, R. W. and Billah, M. M. (1988) J. Biol. Chem. **263**, 12472–12477
51 Warnock, D. E., Roberts, C., Lutz, M. S., Blackburn, W. A., Young, W. W. and Baenziger, J. U. (1993) J. Biol. Chem. **268**, 10145–10153
52 Stow, J. L., de Almeida, J. B., Narula, N., Holzman, E. J., Ercolani, L. and Ausiello, D. A. (1991) J. Cell Biol. **114**, 1113–1124
53 Muller, L., Picart, R., Barret, A., Bockaert, J., Homburger, V. and Tougard, C. (1995) Mol. Cell. Neurosci. **5**, 556–566
54 Bennett, J. P., Cockcroft, S., Caswell, A. H. and Gomperts, B. D. (1982) Biochem. J. **208**, 801–808
55 Kusner, D. J., Schomisch, S. J. and Dubyak, G. R. (1993) J. Biol. Chem. **268**, 19973–19982
56 Whatmore, J., Cronin, P. and Cockcroft, S. (1994) FEBS Lett. **352**, 113–117
57 Cockcroft, S., Taylor, J. A. and Judah, J. D. (1985) Biochim. Biophys. Acta **845**, 163–170
58 D'Souza-Schorey, C., Li, G., Colombo, M. I. and Stahl, P. D. (1995) Science **267**, 1175–1178
59 Rawler, A. J., Roelofsen, B., Wirtz, K. W. A. and Op den Kamp, J. A. F. (1982) FEBS Lett. **148**, 140–144
60 Helms, J. B., De Vries, K. J. and Wirtz, K. W. A. (1991) J. Biol. Chem. **266**, 21368–21374
61 Olivos-Glander, I. M., Janne, P. A. and Nussbaum, R. L. (1995) Am. J. Hum. Genet. **57**, 817–823
62 Zhang, X., Jefferson, A. B., Auethavekiat, V. and Majerus, P. W. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 4853–4856
63 Terui, T., Kahn, R. A. and Randazzo, P. A. (1994) J. Biol. Chem. **269**, 28130–28135
64 Randazzo, P. A. and Kahn, R. A. (1994) J. Biol. Chem. **269**, 10758–10763
65 Cockcroft, S., Howell, T. W. and Gomperts, B. D. (1987) J. Cell Biol. **105**, 2745–2750
66 Harlan, J. E., Hajduk, P. J., Yoon, H. S. and Fesik, S. W. (1994) Nature (London) **371**, 168–170
67 Janmey, P. A. (1994) Annu. Rev. Physiol. **56**, 169–191
68 Leyte, A., Barr, F. A., Kehlenbach, R. H. and Huttner, W. B. (1992) EMBO J. **11**, 4795–4804
69 Colombo, M. I., Inglese, J., D'Souza-Schorey, C., Beron, W. and Stahl, P. D. (1995) J. Biol. Chem. **270**, 24564–24571
70 Franco, M., Paris, S. and Chabre, M. (1995) FEBS Lett. **362**, 286–290
71 Gelas, P., Ribbes, G., Record, M., Terce, F. and Chap, H. (1989) FEBS Lett. **251**, 213–218

Received 1 April 1996/29 July 1996; accepted 19 August 1996