ADP ribosylation factor 1 mutants identify a phospholipase D effector region and reveal that phospholipase D participates in lysosomal secretion but is not sufficient for recruitment of coatomer I

David H. JONES*, Ben BAX†, Amanda FENSOME* and Shamshad COCKCROFT*[1]

*Department of Physiology, Rockefeller Building, University College London, University Street, London WC1E 6JJ, U.K., and †Department of Crystallography, Birkbeck College, Malet Street, London WC1E 7HX, U.K.

The small GTP-binding protein, ADP-ribosylation factor 1 (ARF1) is essential for the formation of coatomer-coated vesicles from the Golgi and is also an activator of phospholipase D (PLD). Moreover, ARF1-regulated PLD is part of the signal-transduction pathway that can lead to secretion. In this study, substitution and deletion mutants of ARF1 were tested for their ability to activate PLD. These map the PLD effector region of ARF1 to the α2 helix, part of the β2-strand and the N-terminal helix and its ensuing loop. ARF mutants with an increased or decreased ability to activate PLD showed similar characteristics when tested for their ability to stimulate secretion from HL60 cells. ARF1, deleted of the N-terminal 17 amino acid residues (Ndel17), did not support PLD activity or secretion, and neither did it inhibit the activity of wild-type myristoylated ARF1 (myrARF1). In contrast, Ndel17 effectively competed with wild-type myrARF1 to prevent coatomer binding to membranes. This appears to define a structural role for Ndel17, as it can bind a high-molecular mass complex in cytosol. In addition, ethanol has no effect on recruitment of coatomer to membrane. We conclude that the function of ARF-regulated PLD is in the signal-transduction pathway leading to secretion of lysosomal granules, and not as an essential component of ARF1-mediated coatomer binding.

Key words: ARF1, ARF6, chimaeras, HL60 cells, membrane traffic.

INTRODUCTION

The ADP-ribosylation factor (ARF) family are a group of myristoylated, small GTP-binding proteins of 21 kDa, which are related in sequence and structure to the Ras superfamily and the heterotrimeric G-protein α-subunits. In eukaryotes, ARF is abundant and ubiquitous, and is highly conserved across phylogenetic lines. The family currently consists of six mammalian ARFs, with homologues found in organisms as diverse as *Arabidopsis*, *Drosophila*, budding and fission yeasts and *Giardia lamblia* [1]. ARF proteins have since been shown to regulate the reversible binding of coat proteins to membranes and have been found on both clathrin-coated and non-clathrin-coated vesicles [2,3]. It is thought that cytosolic ARF-GDP interacts with a brefeldin A-sensitive ARF guanine nucleotide exchange factor, resulting in nucleotide exchange and membrane binding. Myristoylation of ARF is not essential for membrane binding of ARF1 [4], but is facilitated by the myristoyl group and also by hydrophobic residues in the N-terminal helix of ARF1 [5–7]. ARF binding allows subsequent recruitment of coat proteins (including coatomer and adaptor proteins, AP1, AP2 and AP3) to initiate vesicle formation. Subsequent uncoating of the vesicle requires that ARF-GTP is hydrolysed to ARF-GDP, allowing fusion with an acceptor membrane [8].

ARF proteins can directly activate phospholipase D (PLD), an enzyme that hydrolyses phosphatidylcholine (PC) to phosphatidic acid (PA) [9,10]. Two PLD enzymes (PLD1 and PLD2) have been cloned from mammalian cells and both isoforms are regulated by ARF proteins [11,12]: PLD1 is activated by as much as 20-fold and PLD2 by 2-fold *in vitro*. It has been proposed that coatomer binding to membranes may be due to ARF-dependent activation of PLD, as cell-lines with a high intrinsic PLD activity do not require cytosolic ARF1 for initiating coat assembly and are insensitive to down-regulation of ARF1 activity by brefeldin A [13–15]. The same authors showed that membranes initially primed with ARF1 (so that PLD is activated) no longer need ARF1 for subsequent coatomer binding, and that formation of coated vesicles was sensitive to ethanol at concentrations that inhibit the production of PA by PLD [14]. However, others have shown that ARF is always present in molar excess over coatomer, and that PA levels do not increase during coat recruitment [16]. Previous suggestions that ARF1 has a structural role in the formation of coated vesicles [17] is supported by recent studies showing that ARF1 can directly interact with the coatomer β-subunit [18].

An additional activity of ARF1 is the ability to restore secretion in HL60 cells, a cell-line which is neutrophil-like [19]. Like neutrophils, these cells contain granules which are modified lysosomes that can undergo secretion after the addition of guanine nucleotides and Ca^{2+} to permeabilized cells [20]. Receptor-stimulated secretion from HL60 cells and neutrophils is inhibited by ethanol, indicating that PLD activation couples events that regulate the secretory machinery [21]. The ability of ARF1 to restore secretion was suggested to be due to ARF1 as an activator of PLD [19]. Additional support comes from the observation that ARF is recruited to neutrophil membranes after activation by *N*-formylmethionyl-leucylphenylalanine [22]. Collectively, these results strongly support the suggestion that in neutrophils and HL60 cells, ARF mediates agonist-dependent PLD activation and, subsequently, secretion.

Abbreviations used: ARF, ADP-ribosylation factor; Ndel17, ARF1 deleted of 17 residues from the N-terminus; PLD, phospholipase D; PEt, phosphatidylethanol; PC, phosphatidylcholine; COPI, coat protomer I; PA, phosphatidic acid; [S]GTP, guanosine 5′-[γ-thio]triphosphate; myrARF1, myristoylated ARF1.

[1] To whom correspondence should be addressed (e-mail s.cockcroft@ucl.ac.uk).

ARF proteins are required for both vesicle biogenesis and for receptor-regulated secretion. To address whether either of these functions of ARF1 are mediated via PLD activation, we have identified the PLD effector region of ARF1. Using a variety of ARF mutants, we report that ARF-restored secretion requires PLD activation, but recruitment of coatomer is not dependent on PLD activation. We conclude that ARF1 has several independent functions, including activation of PLD activity and recruitment of protein coats for vesicle biogenesis.

METHODS AND MATERIALS

DNA manipulations and mutagenesis

DNA manipulations were performed in *Escherichia coli* XL1 Blue, and protein expression was performed in *E. coli* BL21 (DE3; pLysS). Myristoylated ARF1 (myrARF1) was expressed in *E. coli* BL21 (DE3; pBB131) [23]. Plasmid pET22b (Novagen) was used as the expression vector to produce native protein as previously described [24]. All mutant proteins behaved in a similar fashion. Mutagenesis was performed by two rounds of the PCR; nucleotide sequences of oligonucleotides used may be obtained from the authors. After ligation into the expression vector, all PCR products were sequenced.

Representation of the GTP-bound form of ARF1

A worm representation was generated based on the program GRASP [25].

Recombinant PLD preparation and its assay *in vitro*

Baculovirus membranes from cells infected with a PLD1-expressing recombinant virus were prepared as described previously [11]. PLD was stored at 4 °C in buffer containing a protease inhibitor cocktail, and this was stable for 2–3 weeks. Freezing PLD preparations resulted in a significant reduction in activity. The PC substrate used in all *in vitro* assays was 1,2-didecanoyl-*sn*-glycero-3-phosphoryl[^{3}H]choline (C_{10}-PC; 85 Ci/mmol) as described previously [26].

Permeabilized HL60 cell assays for PLD activation

HL60 cells were labelled for 48 h with [*methyl*-^{3}H]choline chloride. The cells (5 ml of cells; 10^7 cells per ml) were permeabilized in Pipes buffer, pH 6.8 (20 mM Pipes/3 mM KCl/137 mM NaCl) supplemented with 1 mg/ml glucose and 1 mg/ml BSA for 10 min with streptolysin O (0.4 unit/ml) in the presence of calcium buffered at 100 nM (with 3 mM EGTA). The cells were diluted to 50 ml with Pipes buffer at 4 °C and centrifuged at 2000 ***g*** for 5 min. The cytosol-depleted cells were resuspended in a small volume of cold Pipes supplemented with MgATP, $MgCl_2$ and calcium buffers. The final concentration in the assay was 1 mM MgATP, 2 mM $MgCl_2$ and calcium at 10 μM in an assay volume of 50 μl. The cells (20 μl) were transferred to Eppendorf tubes on ice containing the proteins and guanosine 5′-[γ-thio]triphosphate ([S]GTP) (10 μM) in 30 μl of Pipes buffer. The samples were transferred to 37 °C for 30 min. At the end of the incubation, the cells were centrifuged (2000 ***g***) at 4 °C and the supernatants (45 μl) were analysed for the release of [^{3}H]choline (PLD activity) as previously described [10].

Permeabilized HL60 cell assays for restoration of secretion

The protocol used for restoration of secretion was exactly the same as for PLD activation, except that the cells were not prelabelled with [^{3}H]choline and 40 μl of the supernatants were analysed for hexosaminidase as described previously [21]. In some experiments (e.g. Figure 6) the assay volume was doubled to 100 μl and secretion and [^{3}H]choline release were measured from the same assay tubes.

[S]GTP-binding assay

The [S]GTP-binding assay was performed using the filter-binding method [27], except that the assay was performed in Pipes pH 6.8, 3 mM KCl, 137 mM NaCl. [^{35}S]GTP was obtained from NEN and was added so as to give approx. 2×10^5 c.p.m. per assay tube. Reactions were performed at 30 °C for 90 min. Binding was also tested under identical conditions as used for the *in vitro* PLD assay (though without radiolabelled PC or PLD and with only 1 μM [S]GTP).

Preparation of cytosol and membrane fractions from HL60 cells

Confluent HL60 cells (50 ml; 5×10^7) were harvested, washed twice with cold buffer A (137 mM NaCl/8.1 mM Na_2HPO_4/2.7 mM KCl/1.5 mM KH_2PO_4/2.5 mM EDTA/1 mM dithiothreitol, pH 7.2) and incubated for 5 min in 5 ml of buffer A with 2 mM di-isopropyl fluorophosphate, a protease inhibitor. Cells were again harvested, resuspended in 1 ml of buffer A with a cocktail of protease inhibitors (final concentrations used: leupeptin, 1 μg/ml; aprotinin, 1 μg/ml; pepstatin A, 1 μg/ml; soybean trypsin inhibitor, 5 μg/ml; benzamidine, 200 μg/ml), sonicated and centrifuged at 2000 ***g*** for 10 min. The supernatant was then centrifuged for 3 h at 120000 ***g*** to separate membrane and cytosol. The membrane fraction was resuspended in 0.5 ml of buffer A (with protease inhibitors) and cytosol was concentrated 2-fold using a centricon spun concentrator. Samples were used within 24 h.

Coatomer binding assay

Assays were carried out on 200 μl samples in siliconized centrifuge tubes. This comprised 100 μl of 2 × sucrose buffer (2 × buffer: 50 mM Hepes/KOH, pH 7.0/250 mM KCl/5 mM $MgCl_2$/2 mM dithiothreitol/0.4 M sucrose) and samples of the following: ARF proteins, 50 μg of membrane protein (250 μg/ml), 200 μg of cytosol (1 mg/ml final concentration), 250 μM GTP or [S]GTP as indicated, energy regeneration system (10 × solution: 1 mg/ml creatine kinase/16.4 mg/ml creatine phosphate/10 mM MgATP) and buffer A to 100 μl. Samples were incubated at 37 °C for 15 min, centrifuged at 120000 ***g*** for 15 min, washed in sucrose buffer and processed for Western blotting. After SDS/PAGE, samples were transferred to PVDF membranes, incubated with appropriate antibodies and detected by enhanced chemiluminescence. Monoclonal antibodies against ARF and the β' subunit of coat promoter I (COPI) have been described previously [28].

Assay of PLD activity on membranes

HL60 cells were grown for 3–4 days in the presence of 2 μCi/ml sodium [^{14}C]acetate. Membranes and cytosol were then harvested as described above, and the cytosol fraction was passed over a Superose 12 gel-filtration column and eluted in 20 mM Pipes, pH 6.8/137 mM NaCl/3 mM KCl to obtain an ARF-free coatomer-containing fraction. Assay conditions were identical with those used for coatomer binding, except that ethanol was included at 2%. Coatomer fraction was added to the same concentration as would have been present in 200 μg of cytosol. After incubation, assays of 200 μl were quenched with 750 μl of chloroform/methanol (1:1, v/v) and lipids were extracted and analysed for phosphatidylethanol (PEt) [28].

Gel filtration of ARF1 deleted of 17 amino acid residues from the N-terminus (Ndel17)

Ndel17 or ARF1 (60 μg) was incubated with 3 mg of rat brain cytosol or buffer (20 mM Pipes, pH 6.8/137 mM NaCl/3 mM KCl) in 200 μl for 30 min at 37 °C. Samples were then immediately applied to a Superose 12 gel-filtration column and eluted in the same buffer. These were then analysed by SDS/PAGE and Western blotting using anti-ARF antibodies as described previously [28]. When cytosol is applied with ARF proteins, ARF elutes slighter later than when ARF is applied alone. This is due to back pressure developing in the column due to the proteins of the cytosol.

RESULTS

Mutational analysis identifies regions of ARF1 that are implicated in interactions with PLD

The crystal structure of ARF1-GDP has established that when compared with Ras, ARF-GDP has an extra N-terminal helix, and an extra β-strand (β2E, residues 42–48) in the switch 1 region [5,6]. When ARF is in the GTP-bound conformation, the β2 and β3 strands shift by some 7 Å, causing conformational changes in switches 1 and 2, to move them closer to the active site, and the loss of the extra β2E strand. In addition, the N-terminal helix is displaced so that it becomes available to bind membrane ([29] and see Figure 1). The ARF1-GDP structure implicated two residues, Ile-46 and Asn-52, as potential sites of protein–protein interaction; these would lie in the switch 1 region of Ras. Mutation of Ile-46 to Asp (I46D) increased [S]GTP-binding 6-fold (Figure 2A), probably because the side-chain of Asp-46 interacts with and polarizes the side-chain hydroxyl of Thr-48 in the GTP configuration, enhancing binding of the magnesium ion. This was tested for PLD activation *in vitro* using hPLD1 expressed in Sf9 cells and this mutant produced an equivalent increase in hPLD1 activation (Figure 2A). In contrast, mutation of Asn-52 to Arg (N52R) (or to residues Asp, Val, Ala, Ile) did not affect [S]GTP binding, but wiped out the ability of ARF1 to activate human PLD1 (hPLD1; Figure 2A). Ile-46 thus is not involved in interactions with PLD, whereas Asn-52 is involved.

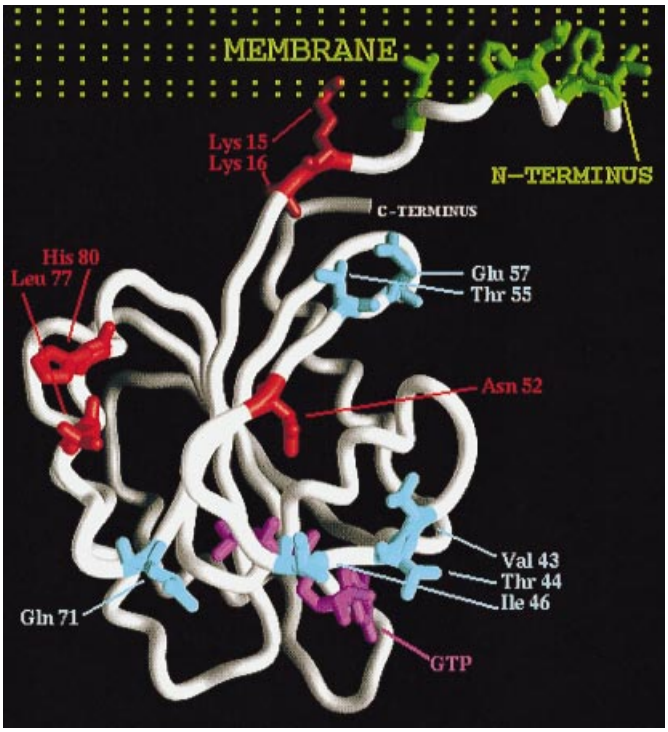


Figure 1 Worm representation of the GTP-bound form of ARF1

Mutations of residues in red disrupt the activation of hPLD1, mutations in blue do not (see Figure 2 for details). The amphipathic N-terminal helix of ARF1 (with hydrophobic residues Ile-4, Phe-5, Leu-8, Phe-9, and Leu-12 shown in green) was modelled in a plausible conformation on to the crystal structure of Ndel17ARF1–guanosine 5′-[β,γ-imido]triphosphate [29]. Val-43, Thr-44 and Ile-46 are in the β2E strand of the GDP form that is lost in the GTP form. Asn-52, Thr-55 and Glu-57 are in the β2 strand. Leu-77 and His-80 are in the α2 helix. Lys-15 and Lys-16 lie in the loop following the N-terminal helix.

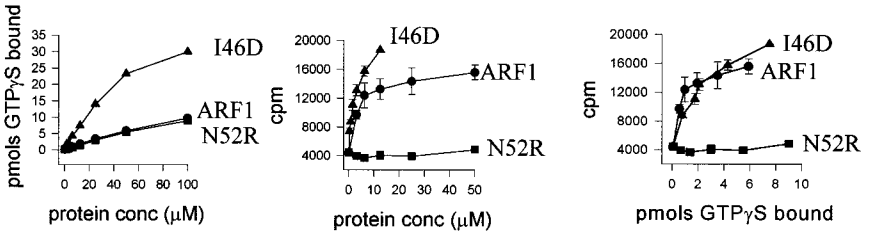
Mutation of both Leu-77 and His-80 (in the α2 helix) to alanine significantly decreased hPLD1 activation (Figure 1 and Figure 2B), while a three-residue deletion at the N-terminus of the α2 helix (deletion of Gln-71, Asp-72 and Lys-73) also decreased hPLD1 activity. However, a Gln-71 to Leu mutant did not affect hPLD1 activity, and neither did the double alanine mutants T55A/E57A, V43A/T44A, suggesting that none of these residues are involved in the interaction with hPLD1.

Deletion of the N-terminal helix of ARF1 increased [S]GTP-binding approx. 40-fold (Figure 2B and [30]), though hPLD1 is not activated by this mutant protein (Figure 2B). The N-terminal helix contains a number of hydrophobic residues and binds membrane lipids when GTP is bound [7]. The N-terminal helix of ARF1 has three pairs of hydrophobic residues compared with ARF6, which has only two pairs (see Figure 2C). Several N-terminal chimaeric proteins of ARF1 and ARF6 were generated to demonstrate that deletion of any hydrophobic residues in this region aids nucleotide exchange (Figure 2C). Presumably this is because the helix can be more readily displaced from the hydrophobic cleft in which it sits in the ARF-GDP conformation. The results shown in Figure 2B and Figure 5A indicate that, whereas any region of ARF6 increases [S]GTP binding, an intact ARF1 N-terminus is required for maximal hPLD1 activation. The ARF1–ARF6 chimaeric protein, which has the N-terminal helix from ARF1 attached to the body of ARF6, is as competent at activating hPLD1 as wild-type ARF1 (Figure 2B). This is in marked contrast to wild-type ARF6, which has a much higher affinity for [S]GTP but a lower ability to activate hPLD1. Since the ARF1 N-terminus on ARF6 is as capable of activating hPLD1 as ARF1, this suggests that differences in the ability of ARF1 and ARF6 to activate hPLD1 are due to differences at the N-terminus. One difference between ARF1 and ARF6 at the N-terminus is at position 15 (K in ARF1, N in ARF6), and a double mutation, K15E/K16E, in ARF1 completely knocked out the ability of ARF1 to activate hPLD1 (Figure 2B). However, ARF1 deleted of the first 13 residues (in which the two lysines are present) was also unable to activate hPLD1. In summary, these results indicate that the N-terminal helix and the ensuing loop, as well as the α2 helix and part of the β2-strand, are important in hPLD1 interaction.

Mutant ARF1 proteins that support PLD activity also reconstitute [S]GTP-dependent secretion

[S]GTP has previously been shown to increase secretion and PLD activity when added to HL60 cells in the presence of the permeabilizing agent, streptolysin O [20]. When HL60 cells are permeabilized for an extended period of 10 min before stimulation with [S]GTP they are no longer responsive. During the period of extended permeabilization, cytosolic proteins, including ARFs, are lost; re-addition of non-myrARF1 was sufficient to restore the [S]GTP-dependent PLD activity and secretory func-

[A] i. GTPγS binding ii. PLD activity iii. PLD activity expressed with respect to GTPγS bound



[B]

Test protein	GTPγS binding	PLD activity	PLD activation with respect to GTPγS bound
ARF1	1	1	1
N52R	1	nil	nil
I46D	6	6	1
Ndel17 - ARF1	40	nil	nil
ARF6	16	0.3	very low
6N - ARF1	18	0.3	very low
1N - ARF6	6	6	1
1Ndel - ARF6	6	0.3	low
K15E/K16E	1	nil	nil
P47/T48/I49 deletion (3 residues)	0.1	nil	nil
T45 to V53 deletion (9 residues)	nil	nil	nil
Q71L	1	1	1
Q71/D72/K73 deletion (3 residues)	0.3	nil	nil
L77A/H80A	0.3	low	low
T55A/E57A	0.3	0.3	1
V43A/T44A	3	3	1

[C] ARF1/ARF6 chimaeric proteins (hydrophobic residues are underlined)

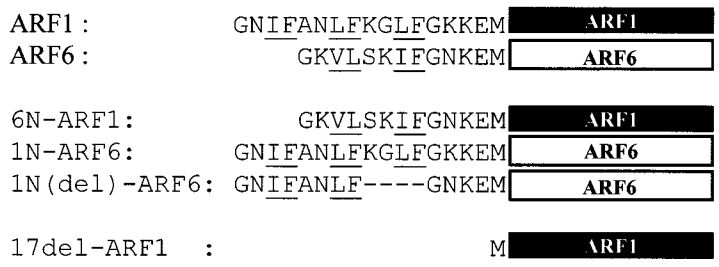


Figure 2 Analysis of regions of ARF1 involved in nucleotide binding and hPLD1 activation

(**A**) (i) [S]GTP-binding to ARF1, N52R and I46D; (ii) hPLD1 activation *in vitro;* and (iii) hPLD1 activity expressed with respect to [S]GTP bound. (**B**) Summary of mutant proteins examined for [S]GTP binding and ability to activate hPLD1. All are compared with the activity of wild-type ARF1 and all are mutations in ARF1 (except the chimaeric proteins described in **C**). The results presented in (**B**) were obtained from several preparations of proteins and the results were reproduced on a minimum of three occasions. (**C**) ARF1/ARF6 chimaeras used in this study. Hydrophobic residues are underlined. GTPγS is [S]GTP.

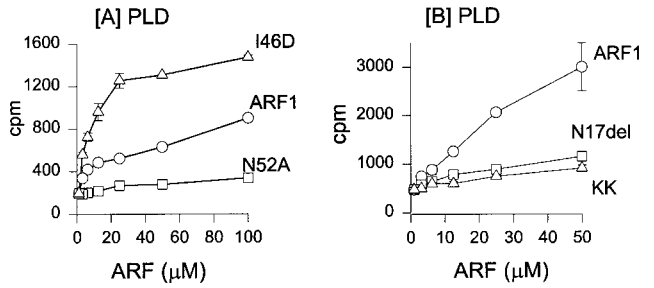


Figure 3 Reconstitution of PLD activity in permeabilized HL60 cells using non-myrARF1 proteins

HL60 cells were prelabelled with [^{3}H]choline for 48 h and the cells were permeabilized with streptolysin O for 10 min to deplete them of endogenous ARF proteins. PLD activity was reconstituted with recombinant ARF proteins in the presence of 10 μM [S]GTP. (**A**) Wild-type ARF1, I46D-ARF1 (a mutant which binds [S]GTP with a 6-fold increased efficiency) and a mutant in a putative effector site for hPLD1 (N52A). (**B**) Wild-type ARF1 compared with Ndel17 and K15E,K16E-ARF1.

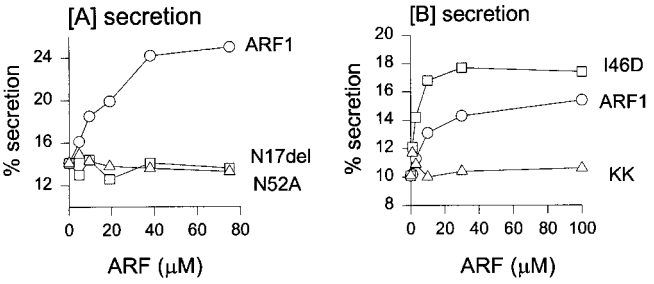


Figure 4 Reconstitution of [S]GTP-stimulated secretion in permeabilized HL60 cells with non-myrARF proteins

HL60 cells were depleted of cytosolic proteins for 10 min by permeabilization with streptolysin O. [S]GTP (10 μM) was used to stimulate secretion in the presence of ARF1 proteins. (**A**) Wild-type ARF1, Ndel17, N52A; (**B**) wild-type ARF1, K15E,K16E and I46D.

tion in ARF-depleted cells [10,19]. We analysed a selection of mutant ARF1 proteins identified above, which have altered effects on either [S]GTP binding or interaction with recombinant hPLD1 (Figure 2B), for activation of PLD and for the restoration of secretory competence in these ARF-depleted HL60 cells.

Mutation of Ile-46 to Asp (I46D, which shows enhanced [S]GTP binding and activation of hPLD1 *in vitro*), was better than wild type in restoring PLD activity (Figure 3) and secretion (Figure 4) in cytosol-depleted HL60 cells stimulated with [S]GTP. The hPLD1 effector site mutant N52A (or mutation to one of Asp, Val, Ala, Ile) restored neither PLD activity nor secretion in permeabilized cells (Figures 3 and 4). The region immediately following the N-terminal helix has also been implicated as an effector site for hPLD1: again, mutation of the Lys–Lys doublet to Glu–Glu did not greatly affect [S]GTP binding, but PLD activity and secretion were not reconstituted by this protein (Figures 3 and 4). All these proteins were tested against wild-type ARF1 and were unable to act as dominant negatives in either secretion or PLD activation (results not shown).

Figure 5 compares ARF1 and ARF6 in their ability to bind [S]GTP, restore secretion and activate hPLD1 *in vitro* in Sf9 membranes. (Identical results were obtained when PLD activation was measured in cytosol-depleted HL60 cells.) Compared with ARF1, ARF6 binds [S]GTP very efficiently, but is a poor activator of hPLD1 and secretion. If the N-terminus of ARF1 is removed and replaced with the N-terminal helix of ARF6, the protein is able to bind [S]GTP with a 6-fold increased potency, and this chimera is better than ARF1 for both PLD activity and secretion (Figure 5). In contrast, the chimaera consisting of the N-terminus of ARF6 on the body of ARF1 is a poor activator of PLD activity and secretion (Figure 5). These results show that PLD activity and secretion from HL60 cells are closely correlated, which implies that a physiological response to PLD activation is the stimulation of secretion.

Inhibitory effects of the N-terminally deleted mutant of ARF1 on recruitment of coatomer but not PLD activity and secretion

ARF proteins recruit coatomer to membranes in the presence of [S]GTP. The role of PLD, if any, in this event is unclear. We wanted to determine if any mutant had dominant negative effects on coatomer recruitment, even though no dominant negatives in PLD activation were identified. We concentrated on ARF1 deleted at the N-terminus because we have recently found that this deletion mutant inhibits the recruitment of paxillin to focal adhesion-like complexes and acts as a dominant-negative mutant [31]. Analysis of the N-terminal helix of ARF1 showed that deletion of this region increases [S]GTP binding but is unable to activate hPLD1 *in vitro* (Figure 2). This protein is unable to

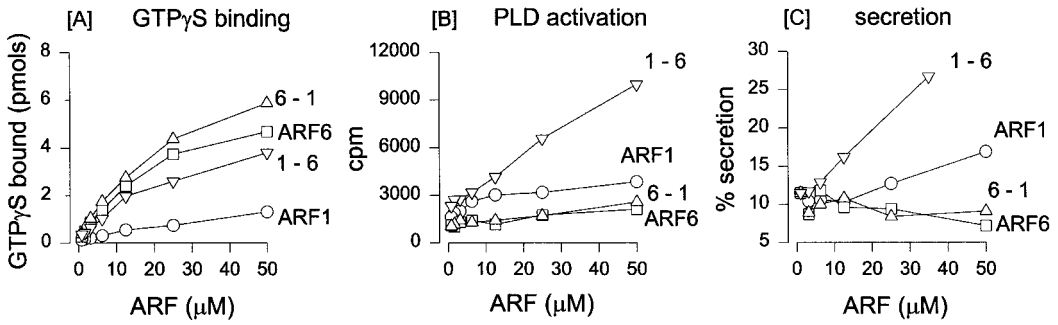


Figure 5 Comparison of (A) [S]GTP binding, (B) hPLD1 activity in Sf9 cells and (C) restoration of secretion in permeabilized HL60 cells

All proteins were produced in *E. coli* and were non-myristoylated. Wild-type ARF1 and ARF6, and two chimaeric proteins were examined. The chimaeras used are: the N-terminus of ARF6 on the body of ARF1 (6–1) and the N-terminus of ARF1 on the body of ARF6 (1–6) (see Figure 2C). GTPγS is [S]GTP.

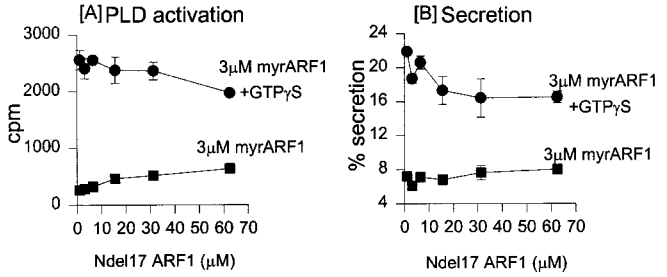


Figure 6 Ndel17-ARF1 does not inhibit (A) PLD activity or (B) secretion from HL60 cells

^{3}H-labelled HL60 cells were depleted of cytosolic proteins for 10 min by permeabilization with streptolysin O. The cytosol-depleted cells were stimulated with 3 μM myrARF1 in the presence of 10 μM [S]GTP in the presence of increasing concentrations of Ndel17-ARF1. GTPγS is [S]GTP.

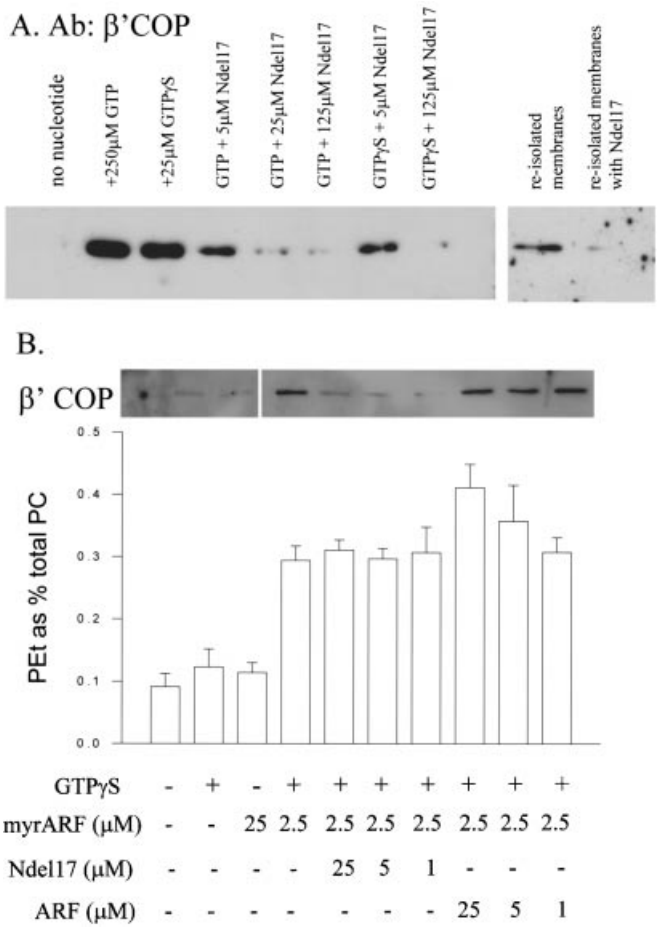


Figure 7 Ndel17-ARF1 prevents binding of coatomer to membranes

(**A**) Recruitment of coatomer to membranes can occur either in the presence of GTP or [S]GTP, and can be competed off with Ndel17. Membranes and cytosol were prepared from HL60 cells. Membranes were incubated with GTP or [S]GTP, cytosol and myrARF1 (25 μM). Ndel17 (5, 25 and 125 μM) was added to the assay in the indicated samples. Ndel17 can strip coatomer from membranes to which coatomer had been pre-bound with myrARF1. The samples were incubated for 15 min at 37 °C for coatomer to be recruited and membranes were harvested. Where the membranes were re-isolated, samples containing membranes, cytosol, myrARF1 and GTP (250 μM) were also incubated for 15 min and again membranes were harvested. The re-isolated membranes were subsequently incubated with or without 25 μM Ndel17 and harvested by centrifugation and the amount of coatomer bound was analysed. (**B**) Ndel17 has no effect on PLD activity but prevents coatomer binding. In contrast, full length non-myrARF1 does not prevent coatomer binding but increases PLD activity. [S]GTP is present at 50 μM and a cytosolic fraction devoid of endogenous ARF was used. GTPγS is [S]GTP.

reconstitute secretion or PLD activity in permeabilized HL60 cells either (Figures 3 and 4). When myrARF1 made in *E. coli* is added to permeabilized HL60 cells at 3 μM there is good activation of PLD activity and secretion (Figure 6). This concentration corresponds to a content of 0.3 μM myrARF1 due to the 10% myristoylation efficiency. When ARF1 deleted of the N-terminus (Ndel17) is titrated into a PLD and secretion reconstitution assay, it does not inhibit either response, even when added to a concentration of 60 μM (Figure 6). However, this mutant protein was found to compete with wild-type myrARF1 in the binding of coatomer to HL60 membranes (Figure 7A). If wild-type myrARF1 and Ndel17 are added together with cytosol in the presence of GTP or [S]GTP, the amount of coatomer which subsequently binds is greatly reduced: Figure 7(A) shows this effect when Ndel17 is present at a 0.2-fold, 1-fold and 5-fold greater molar ratio over myrARF1. The ability of Ndel17 to remove coatomer from membranes could be demonstrated when the order of addition was changed. Thus, if HL60 membranes which have had coatomer pre-bound are then incubated with Ndel17 (25 μM) and re-isolated, coatomer binding is greatly reduced compared with control (Figure 7A).

PLD activity may be monitored in membranes at the same time as coatomer binding. In Figure 7(B), HL60 membranes were incubated with the fraction of cytosol containing coatomer but not ARF. A low concentration of ARF may then be added back to observe effects at sub-saturation concentrations of ARF; any effect on PLD activity should be clear under these conditions. Again coatomer only binds membrane in the presence of 2.5 μM myrARF and nucleotide. Addition of Ndel17 has no effect on PLD activity, but coatomer binding is reduced. Addition of non-myrARF further increases the PLD activity, but does not reduce coatomer binding.

Ndel17-ARF may function by directly interacting with coatomer subunits to inhibit ARF-dependent COPI recruitment. Ndel17 was therefore incubated in the presence or absence of rat brain cytosol at 37 °C for 30 min. The sample was then gel filtered over a Superose 12 column and fractions containing Ndel17 were determined (shown in Figure 8). In the absence of cytosol, Ndel17 eluted as expected in fractions 8 and 9 (corresponding to molecular-mass range 9–40 kDa). However, if Ndel17 was first incubated with cytosol, it eluted in fractions 8/9, but also in the column break-through fraction, fraction 4 (molecular mass > 700 kDa). Coatomer also elutes from the column in this fraction (results not shown). Endogenous myrARF in the cytosol may be seen above the Ndel17 band in fractions 8/9, but this does not appear in the high-molecular-mass fraction, even after a longer exposure than is shown in Figure 8. Furthermore, if non-myrARF1 is incubated with cytosol, this elutes only in fraction 8/9.

One way of diminishing the formation of PA stimulated by the ARF-dependent PLD pathway is to use ethanol to divert the PA to form PEt. We therefore added ethanol to examine whether recruitment of coatomer could be inhibited. Figure 9 illustrates that ethanol titrated between 2 and 6% had a marginal effect on recruitment of coatomer. These results suggest that ARF1 plays a structural role in coatomer recruitment, and PLD alone is not sufficient for coatomer binding.

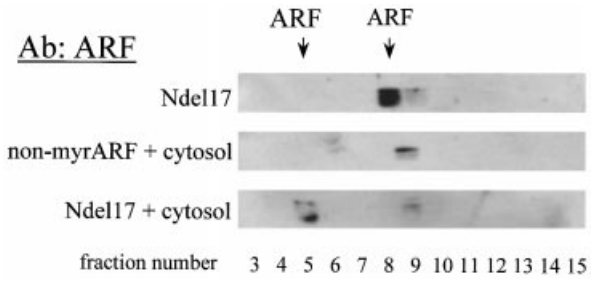


Figure 8 Ndel17 associates with a high-molecular-mass component of rat brain cytosol

Ndel17-ARF was chromatographed by gel filtration in the presence or absence of cytosol. In the presence of cytosol, a fraction of Ndel17 elutes with the break-through fraction on gel filtration. This is not seen when Ndel17 is gel filtered alone, or when full-length non-myrARF is incubated with cytosol and gel filtered. Ab, antibody.

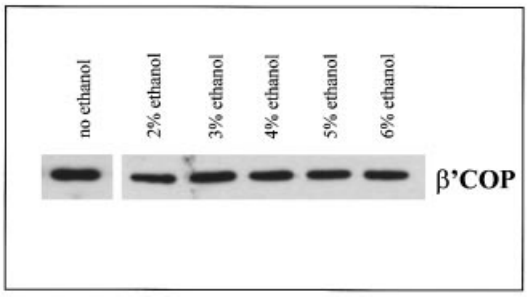


Figure 9 Ethanol does not significantly inhibit binding of coatomer to membranes

HL60 membranes were incubated with cytosol, [S]GTP (50 μM) and myrARF1 (25 μM) in the presence of the indicated concentration of ethanol for 15 min. Membranes were harvested and blotted for coatomer binding. β'COP, β' subunit of COPI.

DISCUSSION

We tested several ARF1 substitution and deletion mutants to identify regions of ARF1 that were central to activating hPLD1 activity using an *in vitro* assay. Mutations map the PLD effector region of ARF1 to the N-terminal helix and the following loop, the α2 helix and part of the β2 strand. Figure 1 identifies the individual residues that affect PLD1 activation (residues marked in red) and defines the face of the molecule that is likely to interact with PLD1. This is in agreement with a recent study [32] that identified a region of ARF1 between residues 34 and 94 as being important in PLD1 activation. Within this region, they identified two residues in the end of the α2-helix that are important in this activation. The crystal structure of ARF, deleted of its N-terminal helix and complexed with the Sec7 domain of ARF exchange factor Gea2 in a nucleotide-free form, has recently been published [29]. The exchange factor domain makes extensive contacts with ARF. These include several residues in the α2 helix and part of the preceding β3 sheet (denoted switch 2) as well as residues 48–53 of ARF and part of the following β2 sheet (switch 1). These are the regions we have shown to be involved in interaction with PLD1, indicating that the effector sites on ARF for PLD and for exchange factors are similar.

From our experimental results, we can dissociate ARF1-dependent coatomer recruitment from ARF-stimulated PLD activation. The mutant Ndel17 does not activate PLD and does not interfere with the activation of PLD by full-length myrARF1. However, it inhibits coat recruitment, indicating that the inhibition is due to a structural effect rather than a catalytic effect on PLD. To lend further support that Ndel17 may interact directly with coatomer, we measured the ability of Ndel17 to bind to a high-molecular-mass component of the cytosol. Although we cannot completely exclude a role for PLD activity in the recruitment of coatomer, ARF1 has additional activities that are essential for binding of coatomer to membranes. Additionally, recruitment of coatomer can occur under conditions of minimal PA production: alcohols that divert PLD-catalysed PA formation to PEt do not inhibit coatomer recruitment. A previous study has used ethanol to provide evidence that PLD mediates ARF-dependent formation of Golgi coated vesicles [14]. It should be stressed that in that study, ethanol inhibited the formation of coated vesicles, but the effect of ethanol on recruitment of coatomer to membranes was not reported.

Additional data that argue against an essential role for ARF1-regulated PLD activity in coatomer recruitment comes from the following observations. ARF-regulated PLD activity is not present in yeast, where ARF-dependent coatomer recruitment occurs normally, and yeast deleted of PLD (known as Spo14) are still able to bud vesicles from the Golgi. In *Saccharomyces cerevisiae*, ARF proteins and Spo14 are required for sporulation, but these effects appear to be independent of each other [33].

ARF1 is also involved in the formation of clathrin-coated vesicles. Three coats have been identified: AP1, AP2 and AP3. The role of ARF1 and PLD in AP1 and AP2 recruitment has been examined, and it has been reported that AP1 recruitment, although ARF-mediated, is also not dependent on PLD [34]. The lack of involvement of PLD activity in coat recruitment is not unexpected, as constitutive membrane trafficking between the Golgi and endoplasmic reticulum occurs when PLD activity would be quiescent. In resting cells, PLD activity is normally low and is rapidly and transiently activated during agonist stimulation of appropriate cell-surface receptors [35].

Our earlier studies suggested that the function of receptor-regulated PLD activity is in signalling to downstream events which, in the case of HL60 cells, is secretion of lysosomally derived granules. To gain further insight, specific mutations were made in ARF1, and these provided tools to study the relationship between ARF1-regulated PLD activity and ARF1-restored secretory competence. It should be stressed that neutrophils and HL60 cells cannot be readily manipulated genetically, ruling out the use of dominant-negative mutants of ARF to study function. The mutation I46D bound [S]GTP with a 6-fold increased efficiency, activated PLD in permeabilized cells with an 8-fold increased efficiency, and reconstituted secretion with a 10-fold increased efficiency. Mutation of Asn-52 to any one of a range of residues resulted in a protein which was unable to activate PLD or secretion, but showed little alteration in [S]GTP binding. Mutation of the lysine doublet at residue numbers 15–16 produced a similar mutant to the Asn-52 mutant, not reconstituting secretion. Thus different nucleotide-binding mutants and different PLD effector mutants have the same effects on PLD activity and on secretion and extend the known correlative effects on PLD and secretion seen when cells are treated with MgATP, Ca^{2+}, PMA and ethanol [21].

ARF proteins, like other monomeric GTPases, clearly have multiple downstream effects in cells. ARF plays a number of distinct roles in the cell, including activation of PLD after

receptor stimulation and formation of several types of vesicles. Although the results presented here argue against an essential role for PLD in recruitment of coatomer, our results do not exclude a possible role for ARF-regulated PLD in other vesicle biogenesis events. ARF1 has been shown to promote vesicle formation from the *trans*-Golgi network, which is not dependent on COPI [36], and this could be PLD-dependent [37].

Note added in proof (received 20 May 1999)

Goldberg [38] has now confirmed that purified coatomer directly interacts with Ndel17–ARF1.

We thank the Wellcome Trust for a programme grant. B.B. and S.C. thank the Biotechnology and Biological Sciences Research Council for funding. We thank Andrew Morris for providing the Sf9 cells expressing PLD1 and Clive Morgan for his advice on the PLD assays. We also thank Jonathan Goldberg for co-ordinates of Ndel17–guanosine 5′-[β,γ-imido]triphosphate–ARF1.

REFERENCES

1 Boman, A. L. and Kahn, R. A. (1995) Trends Biochem. Sci. **20**, 147–150
2 Orci, L., Palmer, D. J., Amherdt, M. and Rothman, J. E. (1993) Nature (London) **364**, 732–734
3 Stamnes, M. A. and Rothman, J. E. (1993) Cell **73**, 999–1005
4 Franco, M., Chardin, P., Chabre, M. and Paris, S. (1993) J. Biol. Chem. **268**, 24531–24534
5 Amor, J. C., Harrison, D. H., Kahn, R. A. and Ringe, D. (1994) Nature (London) **372**, 704–708
6 Greasley, S. E., Jhoti, H., Teahan, C., Solari, R., Fensome, A., Thomas, G. M. H., Cockcroft, S. and Bax, B. (1995) Nature Struct. Biol. **2**, 797–806
7 Antonny, B., Beraud-Dufour, S., Chardin, P. and Chabre, M. (1997) Biochemistry **36**, 4675–4684
8 Tanigawa, G., Orci, L., Amherdt, M., Ravazzola, M., Helms, J. B. and Rothman, J. E. (1993) J. Cell Biol. **123**, 1365–1371
9 Brown, H. A., Gutowski, S., Moomaw, C. R., Slaughter, C. and Sternweis, P. C. (1993) Cell **75**, 1137–1144
10 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
11 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
12 Lopez, I., Arnold, R. and Lambeth, J. D. (1998) J. Biol. Chem **273**, 12846–12852
13 Ktistakis, N. T., Brown, A., Sternweis, P. C. and Roth, M. G. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 4952–4956
14 Ktistakis, N. T., Brown, H. A., Waters, M. G., Sternweis, P. C. and Roth, M. G. (1996) J. Cell Biol. **134**, 295–306
15 Kun, B., Roth, M. G. and Ktistakis, N. T. (1997) Curr. Biol. **7**, 301–307
16 Stamnes, M., Schiavo, G., Stenbeck, G., Sollner, T. H. and Rothman, J. E. (1998) Proc. Natl. Acad. Sci. U.S.A. **95**, 13676–13680
17 Rothman, J. E. (1994) Nature (London) **372**, 55–63
18 Zhao, L., Helms, J. B., Brugger, B., Harter, C., Martoglio, B., Graf, R., Brunner, J. and Wieland, F. T. (1997) Proc. Natl. Acad. Sci. U.S.A. **94**, 4418–4423
19 Fensome, A., Cunningham, E., Prosser, S., Tan, S. K., Swigart, P., Thomas, G., Hsuan, J. and Cockcroft, S. (1996) Curr. Biol. **6**, 730–738
20 Stutchfield, J. and Cockcroft, S. (1988) Biochem. J. **250**, 375–382
21 Stutchfield, J. and Cockcroft, S. (1993) Biochem. J. **293**, 649–655
22 Fensome, A., Whatmore, J., Morgan, C. P., Jones, D. and Cockcroft, S. (1998) J. Biol. Chem. **273**, 13157–13164
23 Duronio, R. J., Jackson Machelski, E., Heuckeroth, R. O., Olins, P. O., Devine, C. S., Yonemoto, W., Slice, L. W., Taylor, S. S. and Gordon, J. I. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 1506–1510
24 Randazzo, P. A., Weiss, O. and Kahn, R. A. (1992) Methods Enzymol. **219**, 362–369
25 Nicholls, A., Sharp, K. and Honig, B. (1991) Proteins **11**, 281–296
26 Vinggaard, A. M., Jensen, T., Morgan, C. P., Cockcroft, S. and Hansen, H. S. (1996) Biochem. J. **319**, 861–864
27 Kahn, R. A. (1991) Methods Enzymol. **34**, 233–242
28 Whatmore, J., Morgan, C. P., Cunningham, E., Collison, K. S., Willison, K. R. and Cockcroft, S. (1996) Biochem. J. **320**, 785–794
29 Goldberg, J. (1998) Cell **95**, 237–248
30 Randazzo, P. A., Terui, T., Sturch, S. and Kahn, R. A. (1994) J. Biol. Chem. **269**, 29490–29494
31 Norman, J. C., Jones, D., Holt, M. R., Barry, S. T., Cockcroft, S. and Critchley, D. R. (1998) J. Cell Biol. **143**, 1981–1995
32 Sung, T., Altshuller, Y. M., Morris, A. J. and Frohman, M. A. (1999) J. Biol. Chem. **274**, 494–502
33 Rudge, S. A., Canenagh, M. M., Kamath, R., Sciorra, V. A., Morris, A. J., Kahn, R. A. and Engebrecht, J. (1998) Mol. Biol. Cell **9**, 2025–2036
34 West, M. A., Bright, N. A. and Robinson, M. S. (1997) J. Cell Biol. **138**, 1239–1254
35 Cockcroft, S. (1997) Prog. Lipid Res. **35**, 345–370
36 Barr, F. A. and Huttner, W. B. (1996) FEBS Lett. **384**, 65–70
37 Chen, Y. G., Siddhanta, A., Austin, C. D., Hammond, S. M., Sung, T. C., Frohman, M. A., Morris, A. J. and Shields, D. (1997) J. Cell Biol. **138**, 495–504
38 Goldberg, J. (1999) Cell **96**, 893–902

Received 23 February 1999/1 April 1999; accepted 27 April 1999