Characterization of the regulation of phospholipase D activity in the detergent-insoluble fraction of HL60 cells by protein kinase C and small G-proteins

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Phospholipase D (PLD) activity has been shown to be GTPdependent both *in vivo* and *in vitro*. One protein that confers GTP sensitivity to PLD activity *in vitro* is the low-molecularmass G-protein ADP-ribosylation factor (Arf). However, members of the Rho family and protein kinase C (PKC) have also been reported to activate PLD in various cell systems. We have characterized the stimulation of PLD in HL60 cell membranes by these proteins. The results demonstrate that a considerable proportion of HL60 PLD activity is located in a detergent-insoluble fraction of the cell membrane that is unlikely to be a caveolae-like domain, but is probably cytoskeletal. This

INTRODUCTION

Phospholipase D (PLD) catalyses the hydrolysis of phosphatidylcholine (PtdCho) to generate phosphatidate and choline [1]. PtdCho-derived phosphatidate is an intracellular messenger that regulates, for example, the oxidative burst in formyl-Met-Leu-Pro-stimulated neutrophils [2], actin-stress-fibre formation in lysophosphatidic acid-stimulated pig aortic endothelial cells [3], and secretion in RBL-2H3 cells [4]. Thus PLD is considered to be an important regulatory enzyme in a diverse range of mammalian cellular processes.

Characterization of PLD in cells and tissues has identified both GTP-dependent and GTP-independent activities [5], although little is currently known about GTP-independent PLD activity. One factor that confers GTP sensitivity to PLD activity in HL60 membranes *in vitro* was identified as the low-molecularmass G-protein ADP-ribosylation factor (Arf) [6,7]. Arf was originally described as a factor required for the efficient ADPribosylation of $G_s \alpha$ catalysed by cholera toxin [8]. Arf is an abundant protein in cells and exists as six isoforms (Arf1–Arf6), allowing the potential for distinct functions and regulatory pathways. Normally, Arf1 is involved in the process of vesicular transport in the Golgi network [9], and it has been shown that PLD is also present in Golgi membranes [10] and is potentially involved in secretion [4].

Other GTP-binding proteins have been implicated in the regulation of PLD activity. In particular, a role for Rho-family members as potential regulators of PLD activity has been highlighted. *In vitro*, RhoA has been shown to confer GTP sensitivity to membrane-bound PLD activities in human neutrophils [11], pig brain and rat brain [12,13]. In cells, the Rho-family members have been demonstrated to play key roles in distinct aspects of cytoskeletal re-organization [14]. However, it is unclear

PLD activity required the presence of Arf1, a Rho-family member and PKC for efficient catalysis of the lipid substrate, suggesting that the activity represents PLD1. We show that recombinant human PLD1b is regulated in a similar manner to HL60membrane PLD, and that PKC α and PKC δ are equally effective PLD activators. Therefore maximum PLD activity requires Arf, a Rho-family member and PKC, emphasizing the high degree of regulation of this enzyme.

Key words: ADP-ribosylation factor, cytoskeleton, phosphatidylcholine, Rho.

whether these roles can be reconciled with the reports of the involvement of PLD in the regulation of cellular shape.

Protein kinase C (PKC) is also involved in the regulation of PLD activity, both *in vivo* and *in vitro*. Phorbol esters stimulate PLD activity in most cell types, and down-regulation of PKC following chronic phorbol ester treatment abolishes agoniststimulated PLD activity [15]. Furthermore, treatment of intact cells with selective PKC inhibitors, such as Ro-31-8220, attenuates agonist-stimulated PLD activity [16]. However, the role of PKC is not clear, since it has been shown that, *in vitro*, PKC α can stimulate PLD activity in the absence of ATP and an activating phorbol ester [17].

Several PLD-encoding genes have been identified in mammalian cells. The *PLD1* gene was originally cloned using degenerate oligonucleotides derived from PLD gene sequences found in yeast and castor beans [18]. Subsequently, two splice variants of the human *PLD1* (*hPLD1*) gene have been identified (termed *hPLD1a* and *hPLD1b*) [19]. *hPLD1b* is shorter than *hPLD1a* because of the removal of a sequence of 114 bases from a region between PLD domains I and II. Recently *PLD2*, a gene distinct from *PLD1*, has been cloned. Analysis of the distribution of hPLD1 and hPLD2 mRNAs has shown that many tissues and cells express both genes [20]. However, HL60 cells predominantly express PLD1, with very little PLD2 [20].

Both cytosolic and membrane-bound PLD activities regulated by low-molecular-mass G-proteins and PKC have been reported in HL60 cells [21,22], although no indication of intracellular location was reported. Here we show that the majority of HL60cell PLD activity is membrane-bound and resistant to extraction with a non-ionic detergent. We also show that the presence of PLD activity in the detergent-insoluble fraction does not correlate with the possibility that this fraction is a glycolipid-enriched membrane (GEM) domain. We demonstrate that the regulation

Abbreviations used: Arf, ADP-ribosylation factor; GEM, glycolipid-enriched membrane(s); GST, glutathione S-transferase; GTP[S], guanosine 5'-[γ-thio]triphosphate; PKC, protein kinase C; PLD, phospholipase D; hPLD1b, human PLD1b; PtdCho, phosphatidylcholine.

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of HL60 PLD activity by low-molecular-mass G-proteins and PKC is similar to the effects of these proteins on the activity of a purified recombinant hPLD1b.

MATERIALS AND METHODS

Materials

RPMI 1640 and heat-inactivated foetal calf serum were from Life Technologies. Lipids were from Lipid Products U.K. and Sigma. Guanosine 5'- $[\gamma$ -thio]triphosphate (GTP[S]) was from Boehringer Mannheim Ltd. [³H]PtdCho (50 Ci/mmol) was from New England Nuclear, and the ECL[®] kit was from Amersham UK Ltd. Mouse monoclonal antibodies to actin and vinculin were from Sigma. Anti-(lamin B) antibody was from Oncogene Science. Goat anti-[glutathione S-transferase (GST)] antibody was from Pharmacia, and rabbit anti-GSANIN antibody was generously donated by Dr. Peter Parker (ICRF, London, U.K.)

HL60 cell culture and fractionation

HL60 cells were cultured in RPMI 1640 medium supplemented with 15 % (v/v) heat-inactivated foetal calf serum, penicillin (100 units/ml) and streptomycin (100 μ g/ml). Cells were harvested at a density of 10^6 cells/ml by low-speed centrifugation (500 g). Cells were lysed by probe sonication on ice in 137 mM NaCl, 8.1 mM KH₂PO₄, pH 7.5, 2.7 mM KCl, 2.5 mM EDTA, 1 mM dithiothreitol and 0.2 mM PMSF, and subjected to ultracentrifugation at 300000 g for 20 min at 4 °C. The supernatant was termed 'cytosol', and the resultant particulate fraction was resuspended in sonication buffer prior to centrifugation at 1000 gto remove unbroken cells and nuclei. The pellet was termed 'lowspeed membranes', and the supernatant was termed 'high-speed membranes'. Triton X-100 was added to high-speed membranes to a final concentration of 1 % (w/v), and the detergent-insoluble fraction was separated from soluble material by ultracentrifugation as before. The detergent-insoluble fraction was washed twice with detergent-free buffer prior to resuspension in the sonication buffer in the absence of detergent.

Recombinant small G-proteins

Epitope-tagged human Arf1, Rac1, RhoA and Cdc42 gene products were expressed in Sf9 cells, extracted, purified by use of the tag and concentrated.

Assay of PLD activity

PtdCho was sonicated with $PtdIns(4,5)P_{2}$ and phosphatidylethanolamine in a buffer containing 125 mM Hepes, pH 7.5, 200 mM KCl, 7.5 mM EGTA and 2.5 mM dithiothreitol. The final concentrations of the lipids in the assays were 8.6 μ M PtdCho (containing approx. 100000 d.p.m. of [3H]PtdCho), $12 \,\mu\text{M}$ PtdIns(4,5)P₂ and $137 \,\mu\text{M}$ phosphatidylethanolamine. HL60 membrane extracts were mixed with 20 μ g of cytosol or $1 \,\mu M$ recombinant low-molecular-mass G-protein, where appropriate, and 30 µM GTP[S] in a final volume of 30 µl. Reactions were started by the addition of 20 µl of sonicated substrate micelle and, following incubation at 37 °C, were terminated by the addition of chloroform/methanol (2:1, v/v). Phases were separated by the addition of chloroform and water. [3H]Choline was separated by Dowex cation-exchange chromatography and quantified by liquid scintillation counting. For the analysis of phosphatidic acid and phosphatidylbutanol production in vitro, [14C]PtdCho replaced [3H]PtdCho in the incubations. Lipids in the chloroform phase were separated by TLC in a solvent

system of chloroform/acetone/acetic acid/methanol/water (50:20:15:10:5, by vol.), and radioactivity was visualized and quantified by phosphorimage analysis.

Preparation of GEM fractions from HL60 cell lysates

GEM fractions were prepared as described [31–34]. Briefly, 0.5×10^8 HL60 cells were lysed in 1 ml of sonication buffer containing 1 % (w/v) Triton X-100 at 4 °C for 30 min. The lysate was diluted with an equal volume of 80 % (w/v) sucrose and mixed thoroughly. The sample was placed in a swing-out centrifuge tube and overlaid with 2 vol. of 30 % (w/v) sucrose and then 1 vol. of 5 % (w/v) sucrose. The samples were centrifuged at 200000 g for 14 h at 4 °C. The GEM fraction was collected from the 5 %/30 % (w/v) sucrose interface, diluted with sonication buffer (in the absence of Triton X-100) and centrifuged at 100000 g for 15 min at 4 °C. The pellets and the GEM fractions were collected, resuspended in sonication buffer and assayed for PLD activity.

Cloning and expression of hPLD1b

The complete cDNA for hPLD1 was isolated from a human placental cDNA library by PCR amplification using primers to the N- and C-termini of PLD1. During the second round of amplification, two nested primers containing *Sal*I and *Not*I sites were used. Amplified DNA was digested with *Sal*I and *Not*I and subcloned into the mammalian expression vector pcDNA3. Both strands from three clones were sequenced in their entirety, using internal primers, to obtain the consensus nucleotide sequence for hPLD1. The isolated PLD1 cDNA encoded a protein of 1036 amino acids with a predicted molecular mass of 119 kDa. This clone was smaller than a *PLD1* gene isolated with the same primers from an HL60 cell library, which encoded a protein of 1074 amino acids. The larger HL60 clone was termed PLD1a, and the smaller placental clone was termed PLD1b.

The hPLD1b gene was subcloned into the baculovirus transfer vector pACGHLT (Pharmingen) between the StuI and NotI sites. This resulted in the generation of an in-frame fusion protein consisting of GST, His, and a protein kinase A consensus phosphorylation site followed by the hPLD1b sequence. The Spodoptera frugiperda cell line, Sf9, was maintained in suspension culture at a density of between 2×10^5 and 2×10^6 cells/ml in stirred vessels at 27 °C in TC100 medium supplemented with 10% (v/v) foetal calf serum. Sf9 cells were co-transfected with 4 μ g of transfer vector and 1 μ g of linearized, polyhedrin-minus viral DNA (Baculogold®; Pharmingen). Homologous recombination between the linearized vector and the transfer vector restored the function of an essential viral gene (ORF1699) to yield infectious recombinant virus. After two rounds of plaque purification, recombinant virus was amplified by large-scale infection until a virus titre of 8×10^7 plaque-forming units/ml was obtained. To generate GST-hPLD1b protein, 500 ml of Sf9 cells at a density of 2×10^6 cells/ml were infected with virus at a multiplicity of infection of 10:1. Cells were harvested after 72 h by centrifugation and lysed, and the fusion protein was captured by glutathione-Sepharose affinity chromatography. Following elution from the resin with glutathione, the GST-hPLD1b protein was concentrated 5-fold. GST-hPLD1b-containing samples were separated by SDS/PAGE (7.5% gels) and either stained with Coomassie Blue or transferred to PVDF membranes, which were then incubated with the goat anti-GST antibody (1:2000 dilution; for 2 h) or the rabbit anti-GSANIN antibody (1:500 dilution; overnight). Immunoreactive bands were detected by incubating membranes with the appropriate horseradish peroxidase-linked secondary antibody followed by ECL. GST-

hPLD1b activity in Sf9 cell lysates was also quantified by an *in vitro* assay.

RESULTS

PLD activity in HL60 membranes

The distribution of PLD activity in HL60 cellular fractions generated by differential centrifugation is shown in Table 1. The specific activity of the PLD in the particulate fraction was approximately twice that in the cytosol, and there was 10 times more PLD activity in the particulate fraction when compared with the cytosol. This contrasts with a report that PLD activity in HL60 cells is predominantly cytosolic [20]. In agreement with previous reports, the addition of cytosol to membranes stimulated PLD activity in a GTP[S]-dependent manner [21].

The HL60 particulate fraction was separated into low- and high-speed membranes by differential centrifugation. The lowspeed membrane fraction, which consisted of unbroken cells and nuclei, contained a PLD of low specific activity that was stimulated 12-fold in the presence of cytosol and represented approx. 5 % of the total PLD present in the particulate fraction. The majority of the PLD activity was found in the high-speed membranes. Addition of cytosol to the high-speed HL60 membrane fraction resulted in a 3-4-fold stimulation of PLD activity. Extraction of the high-speed membrane fraction with the nonionic detergent Triton X-100 (1%, w/v) generated detergentsoluble and -insoluble fractions. Very little PLD activity was detected in the detergent-soluble fraction following removal of the Triton X-100 (results not shown). The detergent-insoluble fraction contained a PLD with low basal specific activity; however, PLD activity was stimulated more than 80-fold by addition of cytosol. Up to 75 % of the PLD activity in cytosolstimulated high-speed membranes was recovered in the detergentinsoluble fraction.

To demonstrate that the detergent-insoluble fraction contained an authentic PLD activity, the effects of primary and secondary alcohols on phosphatidic acid production from [¹⁴C]fatty-acidlabelled PtdCho was examined (Table 2). Phosphorimage analysis of the products, separated by TLC, showed that there was considerably less phosphatidic acid production in the presence of butan-1-ol than in the presence of butan-2-ol. Additionally, the production of phosphatidylbutanol was dependent on the presence of the primary alcohol (Table 2). The production of [³H]choline from [³H]PtdCho catalysed by PLD in the particulate fraction or the detergent-insoluble fraction was unaffected by the

Table 2 Effects of alcohols on phosphatidic acid production

The PLD activity in the detergent-insoluble fraction of HL60 cells was incubated with cytosol and micelles containing [¹⁴C]fatty-acid-labelled PtdCho in the presence of the alcohols indicated. The lipids were extracted, separated by TLC and quantified by phosphorimage analysis. Phosphatidylbutanol and phosphatidic acid were identified using authentic co-migrating standards. Results presented are means (range), and similar data were obtained in two other experiments.

Lipid production (image units)		
Phosphatidic acid	Phosphatidylbutanol	
23061 (1838)	60549 (4219)	
	Lipid production (image L Phosphatidic acid 23061 (1838)	

Table 3 Distribution of Arf-responsive PLD activity in HL60 cell particulate fractions

PLD activity was determined in the fractions alone and in the presence of 1 μ M Arf1. Data are presented as means from duplicate determinations; similar results were obtained with two other preparations. Values in parentheses indicate the fold increase in PLD activity compared with the control (fractions alone).

	PLD activity (pmol/min per mg)				
Fraction	Fraction alone	+ Cytosol	$+1 \mu \text{M}$ Arf1		
Particulate Low-speed membranes High-speed membranes Detergent-insoluble fraction	8.35 0.95 2.65 1.53	23.74 (2.96) 15.07 (21.6) 28.84 (11) 86.6 (67)	14.5 (1.84) 0.4 (0.5) 11 (4.17) 7 (5)		

presence of butan-1-ol or butan-2-ol (results not shown). The results suggest that the majority of the PLD activity is located in the detergent-insoluble fraction of the HL60 membranes. Furthermore, factors in the membranes or the cytosol of HL60 cells are required to maintain the observed PLD activity.

Arf was the first cytosolic factor to be identified that could mediate GTP-dependent PLD activation [5,6]. We found that $1 \mu M$ Arf1 was the optimally effective PLD-activating concentration (results not shown). Recombinant Arf1 ($1 \mu M$) stimulated PLD activity in the HL60 particulate fraction (Table 3). In

Table 1 Distribution of PLD activity in HL60 cells

HL60 cells were harvested by low-speed centrifugation and lysed by sonication. The cell fractions were isolated by differential centrifugation. PLD activity was determined in the fractions alone and in the presence of cytosol. Results are presented as means from duplicate determinations; similar results were obtained with three other preparations.

Fraction	PLD activity (pmol/min per mg)	Total protein (mg)	Total PLD activity (pmol/min)	Stimulation by cytosol (fold)
Particulate	20.02	31.5	630	_
Cytosol	8.48	5	42	_
Particulate + cytosol	39.19	_	1233	1.95
Low-speed membranes	1.39	9.8	13.7	_
Low-speed membranes + cytosol	17.64	_	173	12
High-speed membranes	10.65	14.6	155	_
High-speed membranes + cytosol	53.28	_	778	5
Detergent-insoluble fraction	1.2	4.2	4.7	_
Detergent-insoluble fraction + cytosol	98.59	_	415	88



Figure 1 Effects of Arf and Rho-family members on PLD activity in HL60 membranes

PLD activity in HL60 membranes was quantified by measuring the release of [³H]choline from [³H]PtdCho in the presence of HL60 cytosol, 1 μ M Arf1 and/or 1 μ M RhoA-family member. Data are presented as means (+range) of duplicate determinations, and similar results were obtained in at least three other experiments.

contrast, 1 μ M Arf1 did not stimulate the PLD activity in the low-speed membranes. However, Arf1 stimulated the PLD activity between 4- and 5-fold in both the high-speed membranes and the detergent-insoluble fraction. Although Arf1 was able to stimulate PLD in the detergent-insoluble fraction, this activation yielded approx. 10% of the total PLD activity measured in the presence of cytosol.

Arf1 and Rho-family members co-operate to activate PLD in HL60 membranes

The detergent-insoluble fraction from HL60 cells was used to characterize cytosolic factors that restored the stimulation of PLD activity. Separation of HL60 cytosol by anion-exchange chromatography generated several distinct fractions that stimulated detergent-insoluble PLD activity in the absence of Arf1 and also co-operated with Arf1 to further enhance PLD activity (results not shown) [11–13]. RhoA and other Rho-family members have been reported to activate HL60 PLD alone and to synergize with Arf [11–13]. These proteins were therefore considered as candidate factors to fully restore the PLD activity present in detergent-insoluble fraction.

The effect of RhoA in combination with Arf1 on PLD activity was initially characterized in the HL60 cell particulate fraction. Arf1 (1 μ M) was an effective activator of PLD. In contrast, 1 μ M RhoA, Rac1 or Cdc42 was unable to stimulate PLD activity (Figure 1). However, the combination of Arf1 and RhoA resulted in a co-operative stimulation of particulate PLD activity. Figure 1 also shows that this effect was not specific to RhoA, because both Rac1 and Cdc42 were able to co-operate with Arf1 to activate the particulate PLD. These data are in contrast with a previous report that suggested that Cdc42 is a less effective activator of HL60 PLD in comparison with RhoA and Rac1 [21].

Figure 2 illustrates the effects of small G-proteins on PLD activity in the detergent-insoluble fraction of HL60 membranes. Arf1 stimulated PLD activity weakly, and it therefore seemed likely that detergent extraction limited the effectiveness of the action of Arf1 upon PLD. RhoA, Cdc42 and Rac1 were unable to stimulate PLD in the detergent-insoluble fraction. These observations could be explained by the reports that RhoA requires a 50 kDa cytosolic protein to stimulate PLD activity in neutrophils [11] and HL60 cells [24]. It is possible that this factor, and potentially Rho, were removed during detergent extraction, thereby limiting the effectiveness of Arf1 in activating PLD.



Figure 2 Effects of Arf1, Rho-family members and PKC on PLD activity in detergent-extracted HL60 membranes

(A) HL60 membranes were extracted with Triton X-100 (Ext. Mem) and PLD activity was quantified by measuring the release of $[{}^{3}H]$ choline from $[{}^{3}H]$ PtdCho in the presence of 1 μ M Arf1, 1 μ M Rho-family member or HL60 cytosol. (B) Recombinant PKC α (0.1 μ M) was included in PLD incubations containing Arf1 and Cdc42. Data are presented as means (+ range) of duplicate determination, and similar results were obtained in at least three other experiments.

However, the combination of recombinant Arf1 and a Rhofamily member restored the PLD activity in the detergentinsoluble fraction (Figure 2), suggesting that only Arf is required in order to observe effects of Rho upon PLD. The combination of two Rho-family members did not support PLD activity in the detergent-insoluble fraction (results not shown). Inclusion of PKCα in incubations containing Arf1 and Cdc42 further stimulated PLD activity in the detergent-insoluble fraction (Figure 2B). This effect occurred in the absence of ATP or phorbol esters, suggesting that the catalytic activity of PKC is not required during PLD stimulation. The effect of PKC α on PLD activity was not dependent upon a particular Rho-family member, since similar results were observed when Rac1 or RhoA was combined with Arf1 to stimulate PtdCho hydrolysis. The use of the combination of recombinant Arf1, a Rho-family member and PKC α provides evidence that the detergent-insoluble fraction contain a considerable proportion of the total HL60 cell PLD activity.

The generation of a detergent-insoluble fraction by Triton X-100 extraction has been widely used to illustrate the association of a variety of signalling proteins with the cytoskeleton of both nucleated and non-nucleated cells. For example, $G_i \alpha$ subunits of heterotrimeric G-proteins have been found associated with the Triton X-100-insoluble cytoskeleton of differentiated HL60 cells [25,26], and small G-proteins have been found in the Triton X-100-insoluble cytoskeleton of megakaryocytes [27]. Similarly, phosphatidylinositol 3-kinase [28], protein phosphatases 1 and



Figure 3 Analysis of HL60 GEM fraction for PLD activity

GEMs were prepared from HL60 cells, as described in the Materials and methods section. Upper panel: PLD activity in HL60 membranes (Mem), GEMs and the residual pellet was quantified by measuring the release of $[^3H]$ choline from $[^3H]$ PtdCho in the presence of HL60 cytosol (Cyt). The results are presented as means (+ range) from duplicate determinations, and similar data were obtained with one other preparation of GEMs. Lower panel: portions of 30 μ g of HL60 membranes (lane A), cytosol (lane B), residual pellet (lane C) and GEMs (lane D) were subjected to SDS/PAGE and Western blot analysis for the distribution of p56^{lym} was obtained in one other experiment.

2A [29] and the Src-tyrosine kinase $p72^{syk}$ [30] have all been found in the Triton X-100-insoluble cytoskeleton of platelets. The distribution of various proteins within the Triton X-100insoluble fraction of HL60 cells was analysed by Western blotting. The fraction contained histones, presumably from nuclei that had burst during preparation, but was enriched in actin (results not shown), as has also been reported in differentiated HL60 cells [26] and platelets [27,28], consistent with an enrichment of the cytoskeleton.

The detergent-insoluble fraction of cells has also been reported to contain functional domains derived from the plasma membrane, termed caveolae in adherent cells [31,32]. This membrane domain is enriched in a similar subset of signalling proteins found in the Triton X-100-insoluble cytoskeleton, including phosphatidylinositol 4-kinase [32], heterotrimeric G-protein subunits [33] and the Src-family tyrosine kinase p56^{lek} [34]. These domains also characteristically contain glycosylphosphatidylinositol-anchored membrane proteins, glycosphingolipids, cholesterol and up to 50% of the agonist-responsive pool of PtdIns $(4,5)P_{2}$ [31]. The particular lipid content of these domains is believed to determine their insolubility in the non-ionic detergent Triton X-100 and their buoyant density in discontinuous sucrose gradients. When purified, these domains have been shown to contain both caveolae and low buoyant density non-caveolar membranes [32]. The latter domain has also been termed a GEM domain [31].

Thus it was considered that the presence of a considerable proportion of HL60 cell PLD activity in the detergent-insoluble fraction could indicate its association with glycolipid-enriched domains derived from the plasma membrane. Therefore GEMs were prepared from HL60 cell lysates in the presence of Triton X-100 (1%, w/v) by ultracentrifugation through discontinuous sucrose gradients [31–34] and *in vitro* PLD activity was determined in both the pellet and the buoyant GEM fraction. GEMs prepared from HL60 cells did not contain PLD activity in the presence of cytosolic activators (Figure 3, upper panel) or Arf1 (results not shown). However, considerable PLD activity was found in the pellet fraction (Figure 3, upper panel), which was stimulated by Arf1 (results not shown). Western blotting



Figure 4 GST-hPLD1b activity in the presence of Arf1, Cdc42 and PKC

GST-hPLD1b activity was quantified by measuring the release of [³H]choline from [³H]PtdCho in the presence of combinations of 1 μ M Arf, 1 μ M Cdc42 and 0.1 μ M PKC α or PKC δ . Data are presented as means (+ range) of duplicate determinations, and similar results were obtained in one other experiment.

analysis showed that the low-density fraction was enriched in the Src-family tyrosine kinase p56^{*lyn*}, consistent with the isolation of a GEM-like detergent-insoluble membrane domain (Figure 3, lower panel) [33].

Cloning, expression and regulation of a hPLD1b fusion protein

The regulation of the HL60 PLD was compared with that of a purified recombinant hPLD1, since this is the predominant PLD isoform in HL60 cells [20]. A placental hPLD1b gene was subcloned into a baculovirus vector containing the GST gene for expression in Sf9 cells. Expression of GST-hPLD1b in Sf9 cells resulted in constitutive PLD activity in the lysates. Following affinity purification and concentration, the specific activity of GST-hPLD1b was equivalent to 0.09 pmol of PtdCho hydrolysed/min per μg of protein. Removal of the GST moiety resulted in a total loss of PLD activity, and it seems likely that the presence of the N-terminal tag maintains the stability of recombinant hPLD1b (M. N. Hodgkin and J. M. Clark, unpublished work). Figure 4 illustrates that, in the presence of 1 µM recombinant Arf1 and 30 µM GTP[S], GST-hPLD1b activity was stimulated approx. 4-fold. This was the maximum activation attainable with Arf1, since addition of up to $5 \,\mu M$ Arf1 resulted in no further increase in GST-hPLD1b activity (results not shown). This is in agreement with previous reports that have shown Arf concentrations of approx. $1 \,\mu M$ to maximally stimulate hPLD1a, hPLD1b and rat PLD1 [18,19,23]. In the presence of $1 \,\mu M$ recombinant Cdc42 and GTP[S], GST-hPLD1b activity was stimulated up to 30-fold. This level of PLD activation was close to maximal for all the Rho-family members tested. Previous analyses of the stimulation of human and rat PLD1 activities have shown that, at $1 \mu M$, RhoA gave near-maximum stimulation of PLD activity [18,19,23]. However, our data also illustrate that Cdc42 is a considerably more potent activator of GST-hPLD1b than is Arf1, in contrast with previous studies on the regulation of recombinant PLD1 by these small Gproteins [18,19,23]. It is unlikely that the presence of the GST moiety interfered with the activation of GST-hPLD1b, since Arf1 was able to bind to GST-hPLD1b (M. N. Hodgkin and M. J. O. Wakelam, unpublished work). These observations support the results obtained in HL60 cell fractions and other reports of

the co-operative effect of small G-proteins on PLD1 activity [11–13,18,19,23].

The effect of PKC α or PKC δ on GST-hPLD1b activity stimulated by Arf1 plus Cdc42 was investigated. Under the specified conditions (1 μ M Arf1), 0.1 μ M PKC α and 0.1 μ M PKC δ each stimulated PLD activity approx. 5-fold (Figure 4). The activation of PLD by the PKC isoforms occurred in the absence of ATP and an activating phorbol ester. The combination of Arf1 and PKC α or PKC δ resulted in an approx. 20-fold increase in GST-hPLD1b activity. The combination of Arf1 and Cdc42 resulted in a 160-fold increase in GST-hPLD1b activity. In the presence of Arf1, PKC α and Cdc42, PLD activity was stimulated approx. 400-fold.

DISCUSSION

A considerable proportion of the total PLD activity in undifferentiated HL60 cells was located in the Triton X-100insoluble fraction. In the detergent-insoluble fraction, PLD activity was weakly stimulated by Arf and was insensitive to stimulation by the Rho-family members alone. In contrast, Rhofamily members activated purified recombinant hPLD1b more potently than did Arf1. However, PLD activity in the detergentinsoluble fraction could be effectively stimulated by the combination of Arf1, a Rho-family member and PKC α . This cooperative effect of the small G-proteins and PKC was not a consequence of the isolation of the detergent-insoluble fraction, since it was also observed using recombinant hPLD1b.

The detection of PLD activity in the detergent-insoluble fraction of undifferentiated HL60 cells suggests that this location may have an important regulatory role in PtdCho hydrolysis. Several other signal transduction proteins, including lipidmetabolizing enzymes, have been reported to be present in the Triton X-100-insoluble cytoskeleton of a variety of cells. Thus G_{α} subunits of heterotrimeric G-proteins have been found in the detergent-insoluble and actin-enriched cytoskeleton of differentiated HL60 cells [25,26], and small G-proteins of the Rho-family have been found in the detergent-insoluble fraction of megakaryocytes [27]. In platelets, Triton X-100-insoluble cytoskeletal fractions enriched in actin have been shown to contain phosphatidylinositol 3-kinase [28], protein phosphatases 1 and 2A [29] and the Src-family tyrosine kinase $p72^{syk}$ [30]. The possibility that HL60 PLD activity within the detergent-insoluble fraction was associated with detergent-insoluble and GEM domains [32] was discounted, as these domains were found not to contain PLD activity. The presence of histones within the detergent-insoluble fraction was probably a consequence of the isolation of this fraction from nucleated cells, rather than from non-nucleated cells such as platelets. Therefore a considerable proportion of the PLD is associated with the Triton X-100insoluble cytoskeleton in HL60 cells. The identification of PLD activity within the actin cytoskeleton may correlate with the report that PLD activity regulates stress-fibre formation in endothelial cells [3].

Arf1 and RhoA co-operated in activating both HL60 PLD and recombinant hPLD1b activities. This co-operative effect was dependent on the presence of Arf1, since two Rho-family members were unable to support PLD activity. However, Rac1 and Cdc42 could replace RhoA in PLD activation, implying that these interactions involve highly conserved regions present in these Rho-family members and PLD. The co-operative effect of small G-proteins on PLD activity was first reported using partially purified PLD activity [12,13], and has subsequently been observed with recombinant hPLD1s [18,19]. However, the recent report of the characterization of a recombinant rat PLD1 showed that Arf3 did not co-operate with RhoA to stimulate PLD activity [23]. This observation was surprising, since rat PLD1 is highly similar to the human protein. The data presented here also emphasize a difference between the regulation of the recombinant PLD1b and HL60 PLD activities. The soluble nature of the recombinant PLD appears to have facilitated the interaction of the Rho-family members. In contrast, stimulation of HL60 PLD activity by Rho-family members required the presence of Arf1. Arf1 may therefore facilitate the interaction between the PtdCho substrate and the membrane-bound PLD, thereby inducing a conformational change that permits the subsequent interaction of the Rho-family members to further stimulate enzyme activity. These data also support the notion that a complex between the PLD, Arf, a Rho-family member and the substrate micelle forms in vitro which then allows the observed effects upon PLD activity.

It is currently unclear how PLD interacts with the lipid micelle. Although it is likely that PLD binds to PtdCho, several reports have shown that the polyphosphoinositides PtdIns $(4,5)P_2$ and PtdIns $(3,4,5)P_3$ are essential for PLD activity both *in vitro* and *in vivo* [35,36], and we have also observed no PLD activity in the absence of PtdIns $(4,5)P_2$. The role of polyphosphoinositides in stimulating PLD activity is complicated further by the observations that PtdIns $(4,5)P_2$ may also regulate GTP-loading of low-molecular-mass G-proteins such as Arf1 and RhoA, either directly or by activating appropriate exchange factors (e.g. ARNO for Arf) [37,38].

PKC plays a critical role in the regulation of cellular PLD activity. The results presented here show that both PKC α and PKC δ were able to stimulate PLD activity *in vitro* in the absence of ATP and a phorbol ester. Previous reports have suggested that only the conventional PKCs PKC α and PKC β are able to activate PLD in this way [12,18,19]. However, novel PKCs, such as PKC δ , contain a rearrangement of the functional domains found in conventional PKCs [39], and the results reflect the possibility that the availability of these domains may be a key point in PKC-dependent PLD regulation. Nevertheless, our data are in agreement with the suggestion that PKCs interact physically with PLD in a complex that may also contain small G-proteins. Recently, a region of the rat *PLD1* gene has been identified that appears to provide an interaction site for PKC [40].

The magnitude of the stimulation of PLD activity seen both in HL60 membranes and with purified hPLD1b in the combined presence of polyphosphoinositides, Arf1, a Rho-family member and PKC suggests that the concerted action of these factors is necessary for complete PLD activation. The data also support the possibility that these factors act on PLD independently [18]. Physiologically, this type of regulation may be essential for PLD, since its substrate, PtdCho, is an abundant phospholipid in cells. The localization of PLD in the detergent-insoluble fraction of HL60 membranes may reveal that it is, in some way, separated from its substrate. The functions of the individual regulators may be to control the localization of PLD so it can access both PtdIns $(4,5)P_{2}$ and PtdCho in response to agonist stimulation. Further studies of the regulation of PLD by its interacting proteins will provide insight into the conformational changes that must occur to activate PLD.

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