The coordinated regulation of phospholipase D by ADP-ribosylation factors and their exchange factors

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A thesis submitted to the University of London for the Degree of Doctor of Philosophy

October 2003
Acknowledgements

I would like to deeply thank my supervisor, Dr Geraint Thomas, for all his help, encouragement and valuable guidance over the past three years.

I am grateful to Prof. Michael Yaffe, Center for Cancer Research, Massachusetts Institute of Technology, for allowing me to work in his lab.

Special thanks to Borja Perez Mansilla and Joseph Murphy for technical assistance.

Special thanks to Gerald Hammond, Cancer Research UK (London), for the purification of the PH domain proteins.

Special thanks to Dr Jean-Marie Chambard for his help in scanning pictures and proof reading.

Special thanks to Dr Rachel Webb for assistance with statistical analyses.

I would like to say thank you to everyone in the Department of Physiology for friendship.

I would like to acknowledge the Medical Research Council for financial support.
Phospholipase D (PLD) is a phospholipid hydrolyzing enzyme, the activation of which has effects on cellular events including cell growth and membrane trafficking in mammalian cells. In a reconstitution assay consisting of permeabilised HL-60 cells (human myeloid leukemic cells), experiments confirmed that members of the ADP-ribosylation factor (ARF) family proteins including ARF1 and ARF6 were efficient PLD activators. However, ARF1 was a stronger activator of PLD than ARF6, a result that was also found in in vitro PLD assays. Moreover, the myristoylated amino terminal α-helix of ARF was essential for the activation of PLD.

The activation of ARF is in turn regulated by a specific family of small guanine nucleotide exchange factors (GEFs) comprising ARNO, GRP-1 and cytohesin-1. The GEFs catalyze the release of bound GDP and its replacement by GTP, leading to ARF activation. Using the reconstitution assay, the PLD activation mediated by ARF was enhanced by the GEFs. However, the GEFs did not improve the stimulation of PLD by ARF in vitro. Interestingly, these GEFs activated PLD with high potency but none of the three GEFs was more potent than the others in their regulation of PLD in cell-based assays. It seems that this pattern of PLD activation does not reflect differential interactions with major phosphoinositides (PIP$_2$ and PIP$_3$) since these molecules bind equally well to the GEFs.

Importantly, PIP$_2$ and PIP$_3$ increased the potency of PLD activation mediated by the coordinated actions of ARF and its exchange factors indicating the involvement of
other pathways in the regulation of PLD *in vivo*. In particular, it emerged that PIP₃ is a more potent activator of PLD than PIP₂ in the presence and absence of ARF-GEFs.
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Abbreviations

APS: ammonium persulfate
ARF: ADP-ribosylation factor
ATP: adenosine-5-triphosphate
BFA: brefeldin A
BSA: serum bovine albumin
°C: degree Celsius
cAMP: cyclic AMP
cGMP: cyclic GMP
cpm: count per minute
DAG: 1,2-diacylglycerol
DGK: diacylglycerol kinase
d.p.m: desintegration per minute
DTT: DL-Dithiothreitol
EDTA: ethylenediamine tetraacetic acid
EGTA: ethylene glycol-bis (β-aminoethyl ether) N, N, N', N'-tetra acetic acid
ER: endoplasmic reticulum
FMLP: N-formylated-methionine-leucine-phenylalanine tripeptide
FPLC: fast protein liquid chromatography
GAP: GTPase activating protein
GEF: guanine exchange factor
GFP: green fluorescent protein
GPCR: G-protein coupled receptors
GSH: reduced glutathione
GST: glutathione-S-transferase
GTP: guanosine-5'-triphosphate
GTPγS: guanosine-5'-O-(3-thiotriphosphate)
HCl: hydrochloric acid
Hepes: N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid
HRP: horseradish peroxidase
IP3: inositol (1,4,5)-triphosphate
IPTG: isopropyl-d-thiogalactopyranoside
kDa: kilo Dalton
LB: Luria Brot
M: molar
MeOH: methanol
MgATP: magnesium adenosine triphosphate
min: minute(s)
MgCl2: magnesium chloride
MMP-9: matrix metalloproteinase-9
MW: molecular weight
NaCl: sodium chloride
NaH2PO4: sodium phosphate monohydrate
(NH4)2SO4: ammonium sulfate
NaN3: sodium azide
NDPK: nucleoside diphosphate kinase
Ni-NTA: nickel-nitrilotriacetic acid
NMT1: N-myristoyltransferase-1
PA: phosphatidic acid
PAF: platelet activating factor
PAGE: polyacrylamide gel electrophoresis
PAPS: adenosine 3'-phosphate 5'-phosphosulfate
PAR: phosphatidylalcohol
PBS: phosphate-buffered saline
PC: phosphatidylcholine
PCR: polymerase chain reaction
PDGF: platelet-derived growth factor
PE: phosphatidylethanolamine
PE: phosphatidylethanolamine
pfu: plaque-forming unit(s)
PG: phosphatidylglycerol
PH domain: pleckstrin homology domain
PI: phosphoinositide
PIP₂: phosphatidylinositol-4,5-bisphosphate
PIP₃: phosphatidylinositol-3,4,5-triphosphate
PI 3-K: phosphoinositide 3-kinase
PI(4)P5K: phosphatidylinositol 4-phosphate 5-kinase
PIVES: piperazine-N, N'-bis[2-ethanesulfonic acid]
PKC: protein kinase C
PLC: phospholipase C
PLD: phospholipase D
PMA: phorbol 12-myristate 13-acetate
PMSF: phenylmethanesulfonyl fluoride
PS: phosphatidylserine
psi: pounds per square inch
PX domain: phox homology domain
RTK: receptor tyrosine kinases
SDS: sodium dodecyl sulfate
SEM: standard error of the mean
Sf: Spodoptera frugiperda
SLO: streptolysin O
TEMED: N, N', N'', N'''-Tetramethylethylenediamine
TBS: Tris-buffered Saline
TPR domain: tetratricopeptide repeat
VSV-G: vesicular stomatitis virus glycoprotein
v/v: volume: volume ratio
wt: wild-type
w/v: weight: volume ratio
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Chapter one

General introduction
1.1. Cell signalling

Correct cell growth and differentiation in multicellular organisms depends on the ability of the cells to communicate with each other. The mechanisms for such communication include secretion of soluble signalling molecules and direct contacts between cells. Generally, extracellular signalling molecules interact with a receptor embedded within the plasma membrane which transduces the signal across the membrane. Then distinct intracellular signal transduction pathways are initiated that lead to the generation of so-called second messengers within the cell. The second messengers then trigger a series of molecular interactions that lead to specific cellular responses.

The molecular mechanisms of signal transduction pathways have been intensely investigated over the years. The purpose of this introduction is to give an overview of the various receptor-mediated signalling pathways involved in the control and regulation of intracellular events such as gene transcription, protein synthesis and transport, protein phosphorylation and phospholipid metabolism. The latter will be discussed in more details with respect to the regulation of an important enzyme named phospholipase D (PLD).

1.2. Signal transduction via cell surface receptors

Cell surface receptors are integral membrane proteins. They have regions that contribute to three basic domains: extracellular domains, transmembrane domains and cytoplasmic domains. Receptors containing extracellular domains expose some residues to the outside of the cell, interact with and bind to ligands. Receptors with transmembrane domains span the membranes hydrophobic stretches of amino acids in
the lipid bilayer. The cytoplasmic domains of cell surface receptors are tails or loops of the receptor that are within the cytoplasm that react to ligand binding by interacting in some way with other molecules, leading to the generation of second messengers. Cytoplasmic residues of the receptor are thus the effector region of the molecule.

There are four major classes of cell-surface receptors: G-protein-coupled receptors (GPCRs), tyrosine kinase-linked receptors, ion-channel receptors, and receptors with intrinsic enzymatic activity such as receptor tyrosine kinases (RTKs) (Figure 1.1).

Ligand binding to ion-channel receptors changes the conformation of the receptor so that specific ions flow through it. The resultant ion movements alter electric potential across the cell membrane. The acetylcholine receptor at the nerve-muscle junction is an example.

Stimulation of tyrosine kinase-linked receptors leads to the formation of a multimeric receptor, which then interacts with and activates one or more cytosolic protein-tyrosine kinases. The receptors for many cytokines, the interferons, and human growth factor are of this type. These tyrosine kinase-linked receptors sometimes are referred to as the cytokine-receptor superfamily.

The GPCRs and the RTKs will be considered in more details in the following sections with a particular stress on the signalling pathways initiated by these receptors.

1.2.1. Signalling through GPCRs

Many different mammalian cell-surface receptors are coupled to a heterotrimeric signal-transducing GTP-binding protein (G protein). Ligand binding to these receptors activates their associated heterotrimeric G protein, which then activates an effector
Figure 1.1. *Four major classes of cell-surface receptors.* Common ligands for each receptor type are listed in parentheses (Taken from Molecular Cell Biology, http://www.ncbi.nlm.nih.gov/books).
enzyme to generate intracellular second messenger (Figure 1.1). All GPCRs contain seven membrane-spanning regions with their N-terminal segment on the exoplasmic face and their C-terminal segment on the cytosolic face of the plasma membrane (Figure 1.2).

Figure 1.2. Schematic diagram of the general structure of G protein-linked receptors. All receptors of this type contain seven transmembrane α-helical regions. (Taken from Molecular Cell Biology, http://www.ncbi.nlm.nih.gov/books).

This large receptor family comprises light-activated receptors (rhodopsins) in the eye and literally thousands of odorant receptors in the mammalian nose, as well as numerous receptors for various hormones and neurotransmitters (α-adrenergic receptors, β-adrenergic receptors). Although these receptors are activated by different ligands and may mediate different cellular responses, they all activate a seemingly similar signaling pathway (Figure 1.3).

All heterotrimeric G proteins contain α, β, and γ subunits. Prior to activation, the α subunit is bound to GDP. Binding of a trimeric G protein to an activated receptor
Figure 1.3. Schematic overview of common signalling pathways downstream from GPCRs and RTKs. SM, second messenger. (Taken from Molecular Cell Biology, http://www.ncbi.nlm.nih.gov/books).
leads to dissociation of GDP, binding of GTP to $G_{\alpha}$, and dissociation of $G_{\alpha}$-GTP from $G_{\beta\gamma}$. $G_{\alpha}$-GTP and $G_{\beta\gamma}$ can specifically interact with effector proteins such as enzymes, ion channels or receptors leading to changes in their activity. There are four general families of heterotrimeric G proteins: $G_{s}$, $G_{i}$, $G_{q}$, and $G_{o}$ (Neer, 1995).

The best-characterized effector regulated by $G_{s}$ and $G_{i}$ proteins is adenylyl cyclase, which catalyzes the formation of cAMP from ATP. For instance, many of the very different tissue-specific responses induced by binding of adrenaline to $\beta$-adrenergic receptors are mediated by a rise in the intracellular level of cAMP, resulting from activation of adenylyl cyclase. As a second messenger, cAMP acts to modify the rates of different enzyme-catalyzed reactions in specific tissues generating various physiological responses such as liberation of glucose and fatty acids, muscle contraction and relaxation.

The other major signalling pathway activated by a GPCR is the phospholipase C (PLC)-mediated pathway. For instance, hormone binding to receptors coupled to $G_{q}$ protein induces activation of the $\beta$ isoform of PLC (Lee et al., 1994) by the general mechanism outlined in Figure 1.3. As a result, two second messengers, IP$_3$ and DAG, are derived from this pathway through the hydrolysis of PI(4,5)P$_2$ by PLC, another important lipid signalling molecules involved in many cellular events. This activating PLC$\beta$s may be via the $\alpha$ or $\beta\gamma$ subunits.

1.2.2. Signalling through RTKs

The RTKs are a family of more than 50 different transmembrane proteins. The ligands for RTKs are soluble or membrane-bound peptide/protein hormones including nerve growth factor (NGF), platelet-derived growth factor (PDGF), fibroblast growth
factor (FGF), epidermal growth factor (EGF), and insulin. Binding of a ligand to this type of receptor stimulates the receptor's intrinsic protein-tyrosine kinase activity, which subsequently stimulates a signal-transduction cascade leading to changes in cellular physiology and/or patterns of gene expression (Figure 1.3).

Activation of the RTK is initiated by ligand binding to the extracellular domain of the receptor followed by the sequential processes of receptor dimerization and autophosphorylation (transphosphorylation) of tyrosine residues in its cytosolic domain (van der Geer et al., 1994). The activated receptor also can phosphorylate other protein substrates.

Unlike GPCRs, which interact directly with an associated G protein, RTKs are linked indirectly to Ras via two proteins, GRB2 and Sos. Ras is an intracellular GTPase switch protein that acts downstream from most RTKs. Like Gsa, Ras cycles between an inactive GDP-bound form and active GTP-bound form. Ras cycling requires the assistance of two proteins, GEF and GAP, whereas Gsa cycling does not. The SH2 domain in GRB2, an adapter protein, binds to specific phosphotyrosines in activated RTKs. The two SH3 domains in GRB2 then bind Sos, a guanine nucleotide exchange factor, thereby bringing Sos close to membrane-bound Ras-GDP and activating its exchange function. Binding of Sos to inactive Ras causes a large conformational change that permits release of GDP and binding of GTP.

All RTKs in mammalian cells appear to induce a kinase cascade that culminates in activation of MAP kinase pathway. This pathway consists of Ras, Raf, Mek, MAP kinase and Rsk. This serine/threonine kinase, which can translocate into the nucleus, phosphorylates many different proteins including transcription factors that regulate
expression of important cell-cycle and differentiation-specific proteins (Moodie et al., 1993).

Like GPCRs, RTKs can activate the PLC pathway. Indeed, one of the first signalling pathways to be identified in RTK signalling involves PLC-γ. Growth factors such as PDGF, EGF, FGF and NGF are known to stimulate turnover of PI(4,5)P₂ by activating PLC-γ1 in a wide variety of cells (Rhee and Choi, 1992).

Another enzyme implicated in signalling by RTKs is the phosphoinositide 3-kinase (PI3K). PI3K catalyzes the addition of phosphate to the 3'-position of phosphatidylinositol (PI). The 3'-phosphorylated products of this enzyme act as second messengers or affect membrane function. PI3K is composed of regulatory (p85) and catalytic (p110) subunits, each of which is a member of a larger family of homologous proteins. PI3K was the first signalling molecule to be shown to associate with RTKs through its two SH2 domains present in the p85 regulatory subunit (Coughlin et al., 1989). Mutation of the p85 binding sites in the PDGF receptor impairs mitogenesis in response to PDGF in some cell types (Valius and Kazlauskas, 1993). Studies with mutant receptors suggest that PI3K is also apparently required for PDGF-induced membrane ruffling and chemotaxis (Wennstrom et al., 1994).

1.2.3. GPCRs and RTKs can activate PLD pathway

Both GPCRs and RTKs have been implicated in the activation of PLD whose regulation and functional significance will be thoroughly addressed below. PLD catalyses the breakdown of a major membrane phospholipid, namely phosphatidylcholine (PC) into phosphatidic acid (PA) and a soluble head group, choline.
GPCRs have been found to interact with and signal through the small GTP-binding proteins of the Ras superfamily such as ARF and Rho proteins. Inhibitors of ARF and RhoA prevent the activation of PLD through a number of GPCRs such as H1 histamine, B2 bradykinin and M3 muscarinic receptors (Mitchell et al., 1998). In addition, solubilized M3 muscarinic receptor coimmunoprecipitates with ARF1/3 or RhoA.

RTKs such as EGF and PDGF receptors were shown to induce PLD activation in certain cell types. This observation suggested that PLD activity could be modulated by tyrosine phosphorylation (Fisher et al., 1991; Ben and Liscovitch, 1989). Furthermore, priming by GM-CSF of the neutrophil PLD response to chemoattractants such as the peptide fMLP, was tyrosine phosphorylation dependent (Bourgoin et al., 1991).

1.3. Biomembranes: lipid composition

As already mentioned earlier, enzymes such as PLC, PLD and PI3K play important roles in cellular metabolism including the degradation of membrane phospholipids to generate crucial second messengers that participate in numerous signalling pathways.

A typical biomembrane is assembled from phosphoglycerides, sphingolipids, and steroids. All three classes of lipids are amphipathic molecules (i.e., they have a hydrophilic and a hydrophobic part).

In phosphoglycerides, a principal class of phospholipids synthesized in the endoplasmic reticulum (ER), fatty acyl side chains are esterified to two of the three hydroxyl groups in glycerol, and the third hydroxyl group is esterified to phosphate. The phosphate group is also esterified to a hydroxyl group on another hydrophilic
compound, such as choline in phosphatidylcholine (PC) (Figure 1.4). The other phosphoglycerides include phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) (Figure 4).

**Figure 1.4.** Some common phosphoglycerides found in membranes. (Taken from Molecular Cell Biology, http://www.ncbi.nlm.nih.gov/books).

PC is usually the most abundant phospholipid in animal and plants, often amounting to almost 50% of the total, and as such it is obviously the key building block of membrane bilayers. In particular, it makes up a very high proportion of the outer leaflet of the plasma membrane. As noted above, PC is susceptible to hydrolysis by phospholipases, enzymes that cleave various bonds in the hydrophilic ends of phospholipids (Figure 1.5).
Figure 1.5. Specificity of phospholipases. Each type of phospholipase cleaves one of the susceptible bonds shown in red. (Taken from Molecular Cell Biology, http://www.ncbi.nlm.nih.gov/books)

PI is an important lipid, both as a key membrane constituent and as a source of essential signalling molecules such as PIP\(_2\) and PIP\(_3\) which mediate lipid-protein interactions. They are especially effective in specific binding to PH domains of cellular proteins. PI is an acidic phospholipid that in essence consists of a phosphatidic acid backbone, linked via the phosphate group to inositol (Figure 1.4).

In summary, cell stimulation triggers an array of signalling events involving the activation of cell-surface receptors, intracellular protein and lipid molecules leading to cellular responses. The remaining of this introduction will focus on the mechanisms of regulation of PLD and its cellular functions.
1.4. PLD pathway: a major signalling event

1.4.1. Origin

PLD was first purified from plants (Wang et al., 1994) and subsequently purified and cloned in a wide array of species such as bacteria (Iwasaki et al., 1994), yeast (Rose et al., 1995, Waksman et al., 1996), and mammals (Hammond et al., 1995). Two mammalian PLD isoforms, PLD1 (Park et al., 1997; Hammond et al., 1995) and PLD2 (Lopez et al., 1998; Colley et al., 1997; Kodaki and Yamashita, 1997), have been identified in human, mouse and rat species.

PLD1 exists as two splice variants, namely PLD1a and PLD1b which are found in various cell types and tissues of all mammals (Katayama et al., 1998; Hammond et al., 1997; Park et al., 1997; Yoshimura et al., 1996). These two splice variants differ by the absence of a 38-amino acid region in the C-terminus in PLD1b (Katayama et al., 1998; Hammond et al., 1997). Both mammalian PLD1a and PLD1b have very similar properties and, on the whole, can be regulated by the same set of molecules (see below). However, it should be noted that there may be significant differences. For example, rat PLD1b was reported to be less sensitive to a member of the Rho family GTPases namely RhoA than rat PLD1a (Yoshimura et al., 1996).

Three splice variants for human PLD2 has also been cloned (Steed et al., 1998) although no functional differences among variants have been described.

1.4.2. Structure

Comparison of the amino acid sequences of human, yeast, plant and bacterial PLD revealed the existence of functional highly conserved regions such as two essential motifs (Freyberg et al., 2003; Exton et al., 2002; Frohman et al., 1999;
Morris et al., 1996). Figures 1.6 and 1.7 show the sequence alignment and the functional domains of the PLDs mentioned above. These two domains consist of conserved histidine (H), lysine (K), and aspartate (D) residues that are required for catalysis (Sung et al., 1997) and are referred to as HKD domains. In addition to the catalytic domains, PX (phox homology) domain and PH (pleckstrin homology) domain have also been identified in human and yeast PLDs. The PX and PH modules have been widely implicated in a variety of protein-protein and protein-lipid interactions (Lemmon, 2003; Kanai et al., 2001; Ponting, 1996). However, these two domains are absent in the plant and bacterial PLDs. In particular, plant PLD differs from the other PLDs in that it has an N-terminal C2 domain, a structural element that usually mediates calcium and phospholipid-binding and which is found in a wide range of signalling proteins (Ponting and Parker, 1996). Outside the conserved regions, the amino and carboxy terminal sequences of PLD have been suggested to promote interaction with protein kinase C (PKC) (Sung et al., 1999a) and membrane association (Frohman et al., 1999) respectively. The mammalian PLDs also contain a PIP2 binding site and other conserved regions of unknown function. Interestingly, human PLD1 (hPLD1) contains a loop sequence between the two HKD domains that is not present in human PLD2 (hPLD2). As illustrated in Figure 1.6 and Table 1.1 analysis of the amino acid sequences of PLD1 and PLD2 indicates 50% sequence similarity while homology between the yeast, plant, bacterial and human PLD is considerably lower (Morris et al., 1996).

A molecular weight of approximately 120 kDa has been found for the recombinant hPLD1 confirming the predicted size of 124 kDa (Hammond et al., 1995)
Figure 1.6. Alignment of human, yeast, plant and bacterial amino acid sequences for PLD enzymes. Conserved amino acids (aa) are shown in red and similar residues are in blue. The abbreviations used are as follows: hPLD1, human PLD1, gi 1125739; SP014, Saccharomyces cerevisiae PLD, gi 954831; RrPLD, Ricinus communis PLD, gi 626007; SaPLD, Streptomyces antibioticus PLD, gi 517155. Alignment was generated by ClustalW and the printing of the multiple alignment output was performed by Boxshade.
Figure 1.7. Domain structure of human, yeast, plant and bacterial amino acid for PLD enzymes. Regions of conserved sequence are shown. The abbreviations used and protein accession numbers are as follows: PX, phox homology domain; PH, pleckstrin homology domain; PLDc, PLD catalytic site (motifs II and IV known as HKD); C2, calcium- and phospholipid-binding domain; hPLD1, human PLD1, AAB49031; hPLD2, human PLD2, NP_002654; Spo14, Saccharomyces cerevisiae PLD, AAA74938; RcPLD, Ricinus communis PLD, AAB04095; SaPLD, Streptomyces antibioticus PLD, BAA03913. The conserved protein domains were obtained by
Table 1.1.  
Comparison of deduced amino acid sequences of PLDs from various species  
The percentage identity of the indicated PLD-deduced amino acid sequences is indicated above the diagonal, and the percentage similarity is below. The abbreviations are as follows: hPLD1, human PLD1; hPLD2, human PLD2; Spo14, Saccharomyces cerevisiae PLD; RcPLD, Ricinus communis PLD; SaPLD, Streptomyces antibioticus PLD. The asterisk (*) indicates that no significant identity and similarity was found. The percentage global amino acid sequence identity and similarity was calculated by pairwise BLAST using BLOSUM62 matrix.

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<thead>
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<th>hPLD1</th>
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<td>SaPLD</td>
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while hPLD2 has a reported molecular weight of 106 kDa based on the amino acid sequence (Kodaki and Yamashita, 1997).

1.4.3. Expression and localization

The expression and subcellular locations of PLD1 and PLD2 vary within tissues and between cell types. For instance, the brain (Brown et al., 1995) and the lung (Okamura and Yamashita, 1994) are the organs with the highest PLD activity. In human, PLD1 is highly expressed in the kidney and the lung. In rat, PLD1b is the major expressed form and is detected at high levels in the kidney, small intestine, colon and liver. PLD1a is mostly expressed in the lung, heart and spleen (Liscovitch et al., 2000; Katayama et al., 1998). In human, high levels of PLD2 was found in the prostate, placenta and thymus, followed by heart, pancreas, kidney and lung (Liscovitch et al., 2000; Steed et al., 1998). In rodents, highest expression was detected in lung, followed by brain, heart and kidney (Liscovitch et al., 2000; Kodaki and Yamashita, 1997; Colley et al., 1997). Moreover, several types of mammalian cells such as human myeloid HL-60 cells, rat fibroblast Rat-1 cells and mouse T-lymphocyte EL4 cells can express one, both, or neither PLD isoform respectively (Gibbs and Meier, 2000). Interestingly, previous studies showed that HL-60 cells express exclusively PLD1 (Marcil et al., 1997; Ohguchi et al., 1997; Saqib and Wakelam, 1997) although PLD2 expression was also found in granulocytic differentiation of HL-60 cells (Nakashima et al., 1998). This particular type of cell line has been much used by others to investigate the regulation of PLD and is the major focus of attention in this thesis.
There is no general agreement about the exact localization of PLD1 and PLD2 in cells because of differences in cell type and methodology (Exton, 2002; Cockcroft, 2001). However, subcellular localization studies have demonstrated that the mammalian PLD1 is generally detected in the following regions: Golgi apparatus, ER, nucleus, endosomes, lysosomes, and plasma membranes (Freyberg et al., 2001; Liscovitch et al., 1999; Toda et al., 1999; Colley et al., 1997). There is also PLD protein present in more specialized vesicles such as histamine granules in mast cells (Brown et al., 1998), glut4-containing vesicles in adipocytes (Emoto et al., 2000) and also in the secretory granules in neutrophils (Morgan et al., 1997). There is no apparent difference in the localization of the PLD1 splice variants (Toda et al., 1999).

By contrast, the mammalian PLD2 is predominantly found in the plasma membrane (Colley et al., 1997) and, in some cases, membrane ruffles (Honda et al., 1999). However, overexpressed forms of PLD2 has been shown to accumulate in the Golgi apparatus, endosomes, lysosomes, and secretory granules (Freyberg et al., 2001). Moreover, a PLD enzyme has been found in caveolae (Czarny et al., 1999), the well-defined cellular structures resembling membrane invaginations (Harder and Simons, 1997). However, the molecular identity of this caveolar PLD enzyme remains to be firmly established although studies tend to favor PLD2.

1.4.4. Functions of PLD activation

All PLD enzymes catalyze the hydrolysis of the most abundant phospholipid in most cell membranes, PC, to generate PA and the polar head group, choline (Figure 1.8). PLD activity towards other types of phospholipid substrates such as PE and PG.
Figure 1.8. Regulation of PLD by multiple signalling pathways.
has mainly been described in plants (Arisz et al., 2003; Pappan and Wang, 1999) and in bacteria (Okawa and Yamaguchi, 1975).

Interestingly, PLD can catalyze a transphosphatidylation reaction in which a primary alcohol is used instead of water as a "non-physiological" substrate for PLD to produce phosphatidylalcohol (PAR) in place of PA. This type of reaction is often erroneously described as being unique to PLD despite being a relatively common feature of several different enzymes. What is unique is the ability to form phosphatidylalcohols and this feature unequivocally reflects PLD activity, as opposed to any other phospholipase activity against PC. The transphosphatidylation reaction has been extensively used to analyze PLD activation in vivo and in vitro assays. The work described in this thesis took advantage of another approach, which is to quantify the release of choline in broken-cell and cell-free preparations.

In addition to its functions in lipid catabolism, PLD is mostly indirectly implicated in other important cellular processes. Indeed, in most mammalian cells, PLD is activated in response to a broad range of agonists including hormones, neurotransmitters, growth factors, cytokines and related molecules involved in intercellular communication (Exton, 1997; Morris et al., 1997). Moreover, environmental stress conditions in plants such as drought (Frank et al., 2000), hyperosmotic stress (Meijer et al., 2002) and wounding (Ryu and Wang, 1996) have also been reported to trigger PLD activity.

The rest of this chapter will only deal with the regulation of PLD in mammalian cells. Thus, the fact that PLD responds to such a wide range of stimuli that use cell-surface receptors implies that PLD participates in signal transduction mechanisms in
cells. Evidence that PLD activation was required for exocytosis in primary neutrophils and mast cells in their related cell lines, HL-60 cells and RBL-mast cells, came from the observation that alcohols blocked exocytosis. A reconstitution system has been used to examine the requirement for ARF proteins, one of the best known activators of PLD, in PLD activation and exocytosis. In cytosol-depleted cells HL-60 cells, both reactions are refractory to stimulation and can be restored upon addition of ARF proteins (Fensome et al., 1996). ARF-reconstituted secretion is blocked by ethanol, further substantiating the dependence on PA derived from the PLD pathway (Way et al., 2000). Furthermore, PLD has also been found to play a role in controlling changes in the actin cytoskeleton. Stimulation of actin stress fibre formation (Cross et al., 1996) and membrane ruffling (Honda et al., 1999) are both dependent on PLD activity. Membrane ruffling is sensitive to 0.5% butanol while actin stress fibre formation is inhibited by expressing the catalytically inactive form of PLD1.

In addition to mediating the effects of PLD, PA is now considered to be an intracellular second messenger signalling molecule which participates and regulates numerous cellular functions, including ligand-induced exocytosis, protein phosphorylation (Fang et al., 2001; Rizzo et al., 2000), respiratory burst (Erickson et al., 1999; McPhail et al., 1995), endocytosis (Shen et al., 2001), Golgi transport (Ktistakis et al., 1996), and modulation of membrane traffic (Manifava et al., 2001; Bi et al., 1997). Many of these processes are mediated by the binding of PA in a highly selective and specific manner. Thus, it appears that PA might function in a manner similar to many other lipid-derived second messenger molecules (PIP2, PIP3, IP3,
DAG, LPA) and may act by promoting the binding of selected targets to specific regions of the cell membrane.

The direct interaction of PA with the cell proteins has been shown in a small group of proteins. Thus, PA is required for agonist-dependent translocation of serine/threonine kinase Raf-1, an essential component of the MAPK cascade, to intracellular membranes (Rizzo et al., 2000). Insulin stimulation of HIREB fibroblasts led to accumulation of Ras, Raf-1, phosphorylated MEK, phosphorylated MAPK and PA on endosomal membranes. Mutations that disrupt Raf-PA interactions prevented recruitment of Raf-1 to membranes. This observation confirmed previous findings demonstrating that treatment with ethanol inhibited phorbol ester-induced translocation of Raf-1 to membranes (Ghosh et al., 1996). Interestingly, the interactions of PA with Raf-1 have been mapped to a 35 amino acid domain within the CR3 region of Raf-1 (Ghosh et al., 1996). In addition to the MAPK signalling pathway, PA may also regulate another signalling cascade involving the mammalian target of rapamycin (mTOR) that governs cell growth and proliferation (Fang et al., 2001). mTor is a protein kinase related to the PI 3 kinase superfamily. The binding of PA to mTor was shown to implicate R2109 near the rapamycin-binding domain of the protein.

The second messenger role for PA has also been demonstrated in neutrophils (Sergeant et al., 2001). These cell express a large number of non-receptor protein tyrosine kinases which participate in many cellular functions induced by many signalling events following cell stimulation. PA has previously been shown to function as an intracellular regulator of protein tyrosine phosphorylation activity since
exogenously added or endogenously generated PA enhanced tyrosine phosphorylation in neutrophils and other cell types (Siddiqui and Yang, 1995). This suggests a possible activation of either a protein tyrosine kinase or the inhibition of a protein tyrosine phosphatase. Interestingly, Sergeant et al. (2001) observed that Fgr, a Src family enzyme, eluted exclusively with the peak of PA-dependent protein tyrosine phosphorylating activity obtained from gel filtration chromatography of leukocyte cytosol. Thus this observation suggests a possible direct interaction between PA and the protein tyrosine kinase activity in intact cells.

Other protein targets for PA have recently been identified including protein phosphatase-1 (PP-1) (Jones and Hannun, 2002), the protein tyrosine phosphatase SHP-1 (Frank et al., 1999), the cyclic AMP-specific phosphodiesterase PDE4A1 (Baillie et al., 2002) and the p47 phox subunit of the NADPH oxidase (Yaffe, 2002).

Finally, it has been suggested that PA also plays a part in the synthesis of another type of phospholipid namely PIP₂ by activating lipid-metabolizing enzymes. (Cockcroft, 2001). This was based on the finding that PA could stimulate the activity of type I PIP 5-kinase (Honda et al., 1999). However, all these cellular responses cannot be exclusively attributed to PLD since PA is also formed by the tandem action of phosphoinositide-specific PLCs and DGKs. It is well-established that many receptors that activate PLD also trigger hydrolysis of PIP₂ by PLC, releasing inositol IP₃ and DAG (Berridge, 1993). The latter is then rapidly phosphorylated to PA by DGK.

Overall, these numerous cellular events underline the complex regulation of PLD and little is known about the upstream events leading from signal-perception at
the cell surface to PLD activation within the cells. Over the years, strong evidence points to the recurrent role of different types of protein kinases and polyphosphoinositides, small GTP-binding proteins such as Rho, RalA and ARF proteins and their respective regulating proteins, in the control of PLD activation in different in vitro and in vivo model systems. In addition to these various activators, protein inhibitors of PLD have been identified (Han et al., 1996; Kim et al., 1996; Lukowski et al., 1996; Geny et al., 1995).

1.5. Regulation of PLD by PKC

Studies of the activation of PLD by phorbol esters in various cell types implicated PKC in its regulation. Inhibitors of PKC reduced agonist activation of PLD activity, although the magnitude of the inhibition was variable, depending on the agonist and cell type (Exton, 1997). Other approaches indicating the involvement of PKC included down-regulation of the enzyme by prolonged treatment with phorbol ester, and overexpression or antisense deletion of specific PKC isoforms (Exton, 1997). In addition, overexpression of phosphoinositide PLC, which generates DAG and activates PKC, resulted in increased PLD activity (Lee et al., 1994).

Interestingly, the activation of PLD by PKC was also observed in the absence of ATP by a non-phosphorylating mechanism (Hammond et al., 1997). In addition, synergistic interaction between PKC and other small G proteins (Rho, ARF) that activate PLD was observed (Hammond et al., 1997).

Based on these observations, it is possible that agonists that activate PLC cause membrane translocation and activation of conventional PKC which, in turn, activates
PLD. The activation of PLD probably occurs because of direct interaction of PKC with PLD rather than direct phosphorylation.

1.6. Regulation of PLD by Rho family proteins

There is much evidence that RhoA, Rac1 and Cdc42Hs activate PLD1, but not PLD2 (Colley et al., 1997; Hammond et al., 1997). For example addition of GTPγS-activated forms of these proteins to recombinant PLD1 in vitro results in its activation. RhoA is the most efficacious, but Rac1 and Cdc42Hs cause significant activation.

The in vivo effects of RhoA on PLD activity have been explored through the use of clostridial toxins (C3 exoenzyme and toxin B) (Schmidt et al., 1996) and also by transfection of dominant negative and constitutively active forms of RhoA and Rac1. However, these agents do not distinguish between direct and indirect effects of these small G proteins on PLD.

1.7. Regulation of PLD by Ras family proteins

Ras does not directly activate PLD, but there is evidence that Ras mediates the activation of PLD induced by v-Src in vivo (Jiang et al., 1995). Furthermore, RalA, a member of the Ras subfamily, has been shown to interact directly with the ARF-responsive, PIP2-dependent PLD1 (Luo et al., 1997).

1.8. Regulation of PLD by ARF family proteins

1.8.1. Role of ARFs

1.8.1.1. Origin

The ADP-ribosylation factors commonly termed ARFs are one of the five subfamilies of the RAS superfamily of small (= 20 kDa) regulatory guanine nucleotide-binding proteins (Takai et al., 2001). In fact, the ARF family comprises the
ARF, ARL, ARD and SAR proteins. The ARF proteins were originally discovered as cofactors required for the ADP-ribosylation of $G_\alpha$ by cholera toxin (Kahn and Gilman, 1986; Kahn and Gilman, 1984).

1.8.1.2. Different classes of ARFs

The ARF proteins are highly conserved across mammalian species (Hosaka et al., 1996). So far six ARF members and their amino acid sequences (deduced from cDNA sequences) have been reported in mammals (Hosaka et al., 1996; Moss and Vaughan, 1995; Tsuchiya et al., 1991) and three members have been identified in yeasts (Lee et al., 1994). Figure 1.9 shows the amino acid sequence alignment for six mouse ARFs and bovine ARF1. Based on their amino acid sequence similarity, the mammalian ARFs are consequently classified into three groups (Table 1.2). ARF1, ARF2 and ARF3 form class I and are 96 ± 0.8% (n = 6) (mean ± S.E.M) identical to one another at the amino acid level. ARF4 and 5 form class II and are 90% homologous to one another sharing 80.3 ± 0.5% (n = 9) amino acid identity with class I ARFs. ARF6 forms class III and is the most distant member of the family as it shares 68.3 ± 0.8% (n = 6) amino acid identity with class I and class II ARFs.

1.8.1.3. Structure and properties

As indicated above, the comparison of the amino acid sequences of ARF proteins from different species has shown that they are conserved in primary structure. Like all small G proteins, ARFs bind guanine nucleotides (GDP, GTP) and therefore adopt two different conformations: an inactive GDP-bound state and an active GTP-bound state (Figure 1.10). As illustrated in Figure 1.9 ARFs have consensus amino acid sequences that are believed to be involved in guanine nucleotide binding and hydrolysis (Moss

44
Figure 1.9. Alignment of amino acid sequences for mammalian ARFs. Identical residues are in red and similar residues are in blue. The consensus sequences for GDP/GTP-binding and GTPase activities are highlighted in green; G2, the site of N-terminal myristoylation after co-translational removal of methionine, is shaded in yellow. Switch 1 (residues 45-54) and Switch 2 (residues 70-80) are shown in boxes. The abbreviations used and GenBank protein accession numbers are as follows: mARF4, mouse ARF4, JC4948; mARF5, mouse ARF5, JC4949; mARF1, mouse ARF1, JC4944; bARF1, bovine ARF1, NP_788826; mARF3, mouse ARF3, JC4947; mARF2, mouse ARF2, JC4946; mARF6, mouse ARF6, JC4950. Alignment was generated by ClustalW and the printing of the multiple alignment output was performed by Boxshade.
**Table 1.2.**

*Comparison of deduced amino acid sequences of ARFs*

The percentage identity of the indicated ARF-deduced amino acid sequences is indicated above the diagonal, and the percentage similarity is below. The abbreviations are as follows: bARF1, bovine ARF1; mARF2, mouse ARF2; mARF3, mouse ARF3; mARF4, mouse ARF4; mARF5, mouse ARF5; mARF6, mouse ARF6. The percentage global amino acid sequence identity and similarity was calculated by pairwise BLAST using BLOSUM62 matrix.

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Figure 1.10. Cyclical activation/inactivation of ARF proteins.
and Vaughan, 1995; Bourne et al., 1991). In addition to their guanine nucleotide binding property, ARFs have a critical N-terminus which is required for interaction with downstream effectors and therefore essential for ARF activities (Randazzo et al., 1995; Kahn et al., 1992). Unlike the other small G proteins including Ras, Rho/Rac/Cdc42 and Rab, ARF proteins can be post-translationally modified at the N-terminal glycine residue (G2) with the 14-carbon saturated fatty acid myristate. This post-translational modification is catalysed by N-myristoyltransferase (Duronio et al., 1990; Kahn et al., 1988). Myristoylation of ARF proteins has been widely reported to be critical for at least three reasons. First, myristoylation increases the nucleotide exchange on ARFs (Franco et al., 1995). Second, myristoylation contributes to the recruitment of ARFs to natural or artificial membrane phospholipids (Franco et al., 1996). Third, myristoylation has been demonstrated to be essential for ARF interactions with regulator and effector proteins (Massenburg et al., 1994; Brown et al., 1993).

Single crystal X-ray diffraction analysis of ARF1 (Goldberg, 1998; Greasley et al., 1995; Amor et al., 1994) and ARF6 (Pasquato et al., 2001; Menetrey et al., 2000) in several forms have revealed important functional regions. Figure 1.11 shows the crystallographic structure of ARF1 bound to GDP and GTP. Indeed, the overall structure of the ARF molecule consists of seven β-strands, six α-helices and twelve connecting loops arranged in the classical Ras fold. As noted, the feature that distinguishes ARFs from the other Ras family GTP-binding proteins is the N-terminus. This region is an extension of 14 residues as shown for ARF1 (Greasley et al., 1995) and forms an α helix that is tightly packed against the core domain of ARF.
Figure 1.11. Crystallographic structure of ARF1 bound to GDP (bovine GDP-ARF1, PDB code: 1RRF) and GTP (mouse GTP-[Δ17]ARF1, PDB code: 1O3Y). In the ARF1-GTP bound form, the N-terminus α-helix has been deleted.
when it is bound to GDP. Upon GTP binding, this myristoylated N-terminal α helix is thought to be mechanically displaced into the solvent and orient to interact with the membrane. Interestingly, sequence differences among ARFs mainly occur near the N-terminus since the alignment of the mammalian ARFs shows that the N-terminus of ARF6 is shorter than that of the other ARFs by four residues (Figure 1.9). This observation indicates a possible differential affinity interaction with downstream effector proteins. In addition to the N-terminal helix, two highly flexible regions defining, or close to, the acceptor site for the γ-phosphate of GTP have been identified. These two regions have been named switch 1, from residues 45-54, and switch 2, from residues 70-80 (Goldberg, 1998) in accord with the known structure-function relationships of other members of the Ras superfamily. Close to the binding site for guanine nucleotides lies a Mg\(^{2+}\) ion. This ligand also constitutes another significant feature in the ARF molecule and is thought to stabilize the binding of GTP by the switch regions. Consequently, since these two switch regions undergo major rearrangements on GTP↔GDP exchange they probably form, along with the N-terminus, the major sites for the interaction of ARF proteins with their cellular regulators and effectors. Specifically, upon guanine nucleotide exchange, the switch regions are thought to restructure, inducing the movement of one loop (between β2 and β3 strands), which displaces the N-terminal helix from its buried position against the protein core (Figure 1.11). As a result, the N-terminus exposes its attached myristate tail which subsequently inserts into the membrane bilayer. Furthermore, as shown in Figure 1.9, the amino acid sequences for switch 1 and switch 2 regions are surprisingly almost identical among the ARF proteins suggesting that they may have
the same conformation and that they may not be readily distinguishable by the various regulatory and effector proteins that interact with them. It is worth noting that sequence differences between the most distantly related ARF isoforms, ARF1 and ARF6, occur outside the switch regions (Figure 1.9).

Thus minor differences found in the N-terminus region and the switch regions must presumably account for all of the reported differences in the localization and function of ARF1 and ARF6. It should be noted that since these two isoforms of ARF are the most extensively studied, especially where side-by-side comparisons are attempted, there is a significant bias in the literature.

1.8.1.4. Localization and functions

In general, all ARF proteins, with the exception of ARF6, are predominantly cytosolic in their inactive, GDP-bound form. Upon GTP-binding, ARFs translocate from the cytosol to the membranes of different cellular compartments. Indeed, ARF1 and ARF3 are mainly localized in the Golgi and ER where they are involved in the GTP-dependent formation of the COPI-coated and clathrin-coated vesicles that act at distinct steps in intracellular membrane transport (Spang, 2002; Peters et al., 1995; Stamnes and Rothman, 1993). Among the class II ARFs, ARF5 also appears to be localized in the Golgi and the endoplasmic reticular Golgi intermediate compartment (ERGIC) but the function of these proteins is unknown. However, ARF4 has been recently reported to regulate cellular PLD2 activity (Kim et al., 2003). Unlike the other two categories of ARFs, ARF6 is permanently enriched in the plasma membrane where it plays a role in the exocytotic and endocytotic pathways and in controlling the
actin cytoskeleton (Boshans et al., 2000; Radhakrishna and Donaldson, 1997; Peters et al., 1995).

In addition to regulating processes related to vesicle formation and therefore intracellular membrane transport, ARFs have a catalytic role in that they activate phospholipid-metabolising enzymes such as phosphoinositide kinases and PLD. Of course these two effects may be related.

Indeed, it has been reported that ARFs, specifically ARF6, are activators of PI(4)P5K, a lipid-metabolising enzyme primarily responsible for the synthesis of PIP2 (Honda et al., 1999). Interestingly, the activation of PI(4)P5K by ARFs strictly requires PA.

Activation of PLD by ARFs was independently discovered by two groups who identified ARF1 and ARF3 as potent activators of PLD, now known to be PLD1 (Cockcroft et al., 1994; Brown et al., 1993). Massenburg et al. (1994) subsequently separated an ARF-stimulated PLD from an oleate-stimulated PLD after solubilization from brain membranes and showed that the ARF-dependent PLD could be activated by all three classes of mammalian ARFs in vitro. The majority of studies of the regulation of PLD by ARFs have been mostly performed in vitro and in a pseudo in vivo system involving the use of permeabilized cells. These model systems revealed that myristoylation of all ARF subtypes greatly increased their potency and efficacy in activating PLD (Brown et al., 1995; Massenburg et al., 1994; Brown et al., 1993) and therefore clearly required the presence of an intact N-terminus on ARFs (Zhang et al., 1995). Subsequently, ARF-dependent PLD activity has been detected in plasma membranes, nuclei, Golgi and cytosol (Exton, 1999).
1.8.1.5. Regulation of PLD through the ARF-dependent pathway is multi-factorial

There is abundant evidence that PLD can be independently activated by various signalling molecules including PKC, Rho-family G proteins, Ca\(^{2+}\) ions, tyrosine kinases and polyphosphoinositides such as PIP\(_2\) and PIP\(_3\) (Exton, 1999; Houle and Bourgoin, 1999; Exton, 1997). As a result, the physiological regulation of PLD by ARF in a cellular context probably requires the participation of several of these PLD stimulatory molecules. Indeed, experiments in vitro and in cells have clearly demonstrated that the activation of PLD resulted from a synergistic of effects of PKC, Rho proteins (RhoA, Rac1, Cdc42) and other unidentified cytosolic factors with ARF. This observation is further complicated by the fact that the activity of each of these PLD regulatory molecules is in turn controlled by other partner proteins. One of these molecules is PIP\(_2\) itself (whose presence in well-established in vitro PLD assays is an absolute requirement) as this phosphoinositide greatly enhances ARF-stimulated PLD activity (Hammond et al., 1997; Brown et al., 1993). Its role in ARF-dependent PLD activation is thought to arise either through direct interaction with PLD or through the recruitment of ARF-regulatory proteins such as ARF guanine nucleotide exchange factors (ARF-GEFs).

1.8.2. Role of the small GEFs in the activation of ARFs

ARFs are regulated by two types of proteins: ARF guanine nucleotide exchange factors (GEFs) or guanine nucleotide exchange proteins (GEP) and ARF GTPase activating proteins (GAPs). The term guanine nucleotide exchange factors (GEFs) will be used from now on throughout this thesis. The ARF-GEFs promote a fast and effective activation of ARFs through the replacement of GDP with GTP. Hence the
ARF-GEFs are key regulators of the biological functions of ARF proteins including the activation of PLD. By contrast, the ARF-GAPs activate the hydrolysis of bound GTP to GDP (Jackson et al., 2000).

In general, the ARF-GEFs are members of the Sec7 family proteins. This family can be subdivided in two major classes, the large (> 100 kDa) ARF-GEFs and the smaller (< 100 kDa) ARF-GEFs, on the basis of sequence similarity, functional differences and sensitivity to a fungal metabolite BFA.

1.8.2.1. Two different classes of ARF-GEFs

The identification of ARF-GEFs was facilitated by the finding that BFA disrupted Golgi trafficking by inhibiting a Golgi-associated guanine nucleotide exchange activity for ARF1 (Donaldson et al., 1992; Helms and Rothman, 1992). The high-molecular-weight (> 100 kDa) ARF-GEFs were first isolated from yeast Saccharomyces cerevisiae. These include Sec7p (Morinaga et al., 1996), Gea1p and Gea2p (Peyroche et al., 1996). Homologs of the yeast Gea1/2 proteins have subsequently been identified in human (GBF1) and plant (GNOM/Emb30p) (Mansour et al. 1998; Shevell et al., 1994) while homologs of yeast Sec7p comprise mammalian p200 (Mansour et al., 1999; Morinaga et al., 1997), BIG1 and BIG2 (Yamaji et al., 2000). All these large ARF-GEFs, with the exception of GBF1, are BFA-sensitive. However, they are all localized to the Golgi in mammalian cells without exception (Nie et al., 2003; Donaldson and Jackson, 2000; Jackson and Casanova, 2000).

Recently, a novel BFA-insensitive ARF-GEF termed ARF-GEP100 of intermediate size (100 kDa) has been reported and this protein may define a new family of ARF-GEFs (Someya et al., 2001).
In contrast to these BFA-sensitive ARF-GEFs, another family of mammalian small ARF-GEFs (<100 kDa) with BFA-insensitive catalytic activity has been identified. These include ARNO-1 (or cytohesin-2) (Chardin et al., 1996), cytohesin-1 (or B2-1 or ARNO-2) (Kolanus et al., 1996), GRP-1 (or ARNO-3 or cytohesin-3) (Franco et al., 1998; Klarlund et al., 1997), cytohesin-4 (Ogasawara et al., 2000) and EFA6 (Franco et al., 1999). In general, the preferred nomenclature employed for the small ARF-GEFs family is ARNO, cytohesin-1 and GRP-1 exactly as listed above.

The amino acid sequences of all ARF-GEFs contain a central Sec7 domain, a region of roughly 200 amino acids responsible for guanine nucleotide-exchange activity (Jackson and Casanova, 2000; Chardin et al., 1996). Figure 1.12 shows the amino acid sequences of ARNO, cytohesin-1 and GRP-1. Structurally, the Sec7 domain shows very similar arrangements of 10 α-helices grouped in two sets of five, which form a hydrophobic groove that is conserved in all ARF-GEFs (Cherfils et al., 1998). The sequences of motifs 1 and 2 in the Sec7 domain, which are highly conserved among ARF-GEFs, contain all of the major residues necessary for ARF interactions, as shown by the crystallization of nucleotide-free ARF1 and a Sec7 domain GEF (Goldberg, 1998). Importantly, motif 1 contains a conserved and critical glutamic acid residue involved in catalysis of guanine nucleotide exchange (Beraud-Dufour et al., 1998).

In addition to the catalytic Sec7 domain, the ARNO/cytohesin-1/GRP-1 family also possess an N-terminal coiled-coil domain of approximately 40 amino acids, a region believed to be involved in homodimerization (Chardin et al., 1996), Golgi targeting (Lee and Pohajdak, 2000) and interactions with specific protein targets (Venkateswarlu, 2003; Klarlund et al., 2001). Furthermore, proteins in this sub-family
Figure 1.12. Alignment of amino acid sequences for mammalian full-length cytohesin-1, GRP-1 and ARNO. Identical residues are in red and similar residues are in blue. The essential glutamate residue (corresponding to Glu 156 of ARNO) is highlighted in yellow. Details of the constructs are as follows: human cytohesin-1, corresponding to GenBank code Q15438; human GRP-1 corresponding to GenBank code NP_004218 in pQE30; ARNO (from Geraint Thomas) corresponding to GenBank code NP_059431. Diglycine (GG) and triglycine (GGG) motifs are shown by asterisks. Alignment was generated by ClustalW and the printing of the multiple alignment output was performed by Boxshade.
contain a C-terminal pleckstrin homology (PH) domain that mediates binding to polyphosphoinositides particularly PIP$_2$ and PIP$_3$ and seems to mediate the activity of GEFs by recruiting them on the membrane surface where the nucleotide exchange reaction is thought to occur (Jackson and Casanova, 2000; Klarlund et al., 1998; Klarlund et al., 1997; Chardin et al., 1996). The PH domain is a protein module of approximately 120 amino acids, which consists of an orthogonal fold of several $\beta$-sheets and a C-terminal $\alpha$-helix (Rebecchi and Scarlatta, 1998). Analysis of the amino acid sequences of the PH domains among the ARNO/cytohesin-1/GRP-1 family indicate they are about 90% identical (Table 1.3). Notably, the large ARF-GEFs lack the PH domain and therefore must rely on other proteins to mediate membrane recruitment. In addition to the PH domain, the small ARF-GEFs contain a C-terminal polybasic sequence adjacent to the PH domain. This C-terminal polybasic domain is also important for membrane association (Macia et al., 2000; Nagel et al., 1998).

Figure 1.13 shows the domain structure of ARNO, GRP-1 and cytohesin-1, the activity of which will be extensively investigated in this thesis.

Besides sharing these common domain structures, ARNO, cytohesin-1, and GRP-1 are closely related in size (45-50 kDa) and are more similar to each other (83. ± 2.0% global sequence identity) (Table 1.4) than any of them is to the more recently discovered cytohesin-4 (Ogasawara et al., 2000).

There is conflicting information on the subcellular localization of the small ARF-GEFs. Indeed, while some groups have demonstrated the membrane association of these proteins (Ashery et al., 1999; Frank et al., 1998), others have found that these proteins are cytosolic and only translocate to the plasma membrane following
Table 1.3.  
Comparison of deduced amino acid sequences of small ARF-GEF PH domains
The percentage identity of the indicated ARF-GEF-deduced amino acid sequences is indicated above the diagonal, and the percentage similarity is below. Comparison of the sequences of the PH domains correspond to residues 208 to 324 of the full-length ARF-GEFs. The percentage global amino acid sequence identity and similarity was calculated by pairwise BLAST using BLOSUM62 matrix.

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<th>ARNO</th>
<th>GRP-1</th>
<th>Cytohesin-1</th>
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<tr>
<td>ARNO</td>
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<td>89</td>
<td>93</td>
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<tr>
<td>GRP-1</td>
<td>91</td>
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<td>Cytohesin-1</td>
<td>93</td>
<td>91</td>
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Figure 1.13. *Domain structure of ARNO, GRP-1 and cytohesin-1*. Regions of conserved sequence are shown. The abbreviations used and protein accession numbers are as follows: Sec7, Sec7 domain; PH, pleckstrin homology domain. Human cytohesin-1, corresponding to GenBank code Q15438; human GRP-1 corresponding to GenBank code NP_004218 in pQE30; ARNO corresponding to GenBank code NP_059431 (from Geraint Thomas). The conserved protein domains were obtained by submitting the PLD amino acid sequences to the SMART server. The C-terminal polybasic domain contains 33 amino acids past the conserved tryptophan (W). This domain binds to acidic phospholipids.
Table 1.4.  
Comparison of deduced amino acid sequences of small ARF-GEFs 

The percentage identity of the indicated ARF-GEF-deduced amino acid sequences is indicated above the diagonal, and the percentage similarity is below. The abbreviations are as follows: human cytohesin-1, corresponding to GenBank code Q15438; human GRP-1 corresponding to GenBank code NP_004218 in pQE30; ARNO corresponding to GenBank code NP_059431 (from Geraint Thomas). The percentage global amino acid sequence identity and similarity was calculated by pairwise BLAST using BLOSUM62 matrix.

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<th>ARNO</th>
<th>GRP-1</th>
<th>Cytohesin-1</th>
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<tr>
<td>ARNO</td>
<td>-</td>
<td>80</td>
<td>83</td>
</tr>
<tr>
<td>GRP-1</td>
<td>90</td>
<td>-</td>
<td>87</td>
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<tr>
<td>Cytohesin-1</td>
<td>92</td>
<td>92</td>
<td>-</td>
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stimulation of cells with growth factors (Li et al., 2003; Venkateswarlu et al., 1999; Venkateswarlu et al., 1998). Furthermore, reports have indicated that the overexpression of ectopic ARNO (Monier et al., 1998) and GRP-1 (Franco et al., 1998) caused fragmentation of the Golgi, suggesting that their function is in the Golgi. This is supported by the observation that all three small ARF-GEFs act on ARF1, which has historically been localized to the Golgi compartment in addition to the cytosol (Peters et al., 1995).

1.8.2.2. Model for the GDP/GTP exchange reaction

The current proposed model for the guanine nucleotide exchange reaction catalyzed by the ARF-GEFs involves several stages in strict sequence (Jackson and Casanova, 2000; Cherfils and Chardin, 1999; Peyroche et al., 1999). First, GEF recognizes the GDP-bound ARF protein and forms a low affinity complex with GDP-ARF. This then leads to the dissociation of GDP from the initial complex (ARF-GDP-GEF) which becomes a transient high affinity GEF-ARF complex. It is likely that the apo-ARF is held in a state very similar to that of the final GTP-form. GTP subsequently occupies the empty guanine-binding site and "locks" the GEF-induced conformational change. The GTP-ARF then rapidly dissociates from the GEF.

Interestingly, this model of guanine nucleotide has provided an explanation for the molecular mechanism of the inhibitory effects of BFA originally observed on some of the ARF-activating GEFs. Biochemical studies have shown that BFA binds the transient complex formed between ARF-GDP and the Sec7 domain, resulting in an abortive ARF-GDP-BFA-Sec7 domain complex (Chardin and McCormick, 1999; Peyroche et al., 1999).
The sequence of events leading to the activation of ARFs by GEFs is still a matter of debate. It is not clear whether the interaction of ARF and ARF-GEF occurs in the cytosol prior to their translocation to the membrane surface or on the membrane following their independent membrane association via the myristoylated N-terminus of ARF and the coiled-coil and PH domains of ARF-GEF. Alternatively, it is possible that ARF-GEF first localizes to the membrane via interaction with polyphosphoinositides and membrane-bound adaptor proteins in response to cell stimulation and then facilitates the recruitment of ARF. Based on these considerations, it is reasonable to assume that the membrane binding of both ARF and its exchange factor is the prerequisite for protein-protein interactions on the grounds that membrane binding reduces the dimensionality of protein diffusion from three to two dimensions. Random collisions, the major factor influencing kinetic on-rates, are of course more frequent because of this reduction.

**1.8.2.3. Differential GEF specificities for ARF isoforms.**

The majority of large ARF-GEFs including Gea1p/Gea2p (Peyroche and Jackson, 2001; Peyroche et al., 1996), Gnom/Emb30 (Steinmann et al., 1999), Sec7p (Chavrier and Goud, 1999; Sata et al., 1998), p200 (Mansour et al., 1999), and BIG1/BIG2 (Togawa et al., 1999) have been shown to act mostly on class I ARF proteins (Morinaga et al., 1999; Togawa et al., 1999; Morinaga et al., 1996). This is probably because class I ARFs have been the most studied of mammalian ARFs for the past 20 years. In addition, Sec7p, GBF1, BIG1/BIG2 and p200 have been reported to exhibit specificity towards class II and class III ARFs (Claude et al., 1999; Morinaga et al., 1999; Togawa et al., 1999; Sata et al., 1998).
However, a current contention exists over the substrate specificity of the small ARF-GEFs towards the three classes of ARFs. Indeed, ARNO (Beraud-Dufour and Robineau, 2001; Macia et al., 2001; Franco et al., 1998; Paris et al., 1997; Chardin et al., 1996), GRP-1 (Klarlund and Czech, 2001; Franco et al., 1998; Klarlund et al., 1998), cytohesin-1 (Knorr et al., 2000; Ogasawara et al., 2000; Moss and Vaughan, 1999; Betz et al., 1998; Franco et al., 1998; Pacheco-Rodriguez et al., 1998; Meacci et al., 1997), and cytohesin-4 (Ogasawara et al., 2000) have generally been shown to stimulate guanine nucleotide exchange on class I ARFs in vitro. In addition, structural studies mostly used ARNO and ARF1 to demonstrate GEF activity (Beraud-Dufour et al., 1998; Mossessova et al., 1998). However, other groups managed to demonstrate that the ARNO/cytohesin-1/GRP-1 proteins also have distinct specificities with respect to other ARF isoforms. Depending on assay conditions, ARNO has been reported to act on ARF6 in vivo (Santy and Casanova, 2001; Santy et al., 2001; Caumont et al., 2000; Frank et al., 1998b) and in vitro (Santy et al., 1999; Frank et al., 1998a), whereas GRP-1 also functions on class II (Klarlund et al., 1998) and class III (Langille et al., 1999) ARFs. Cytohesin-1 also stimulates guanine nucleotide exchange on ARF5 (Ogasawara et al., 2000) and ARF6 (Knorr et al., 2000; Ogasawara et al., 2000) while cytohesin-4 can activate ARF5 but not ARF6 (Ogasawara et al., 2000). The recently identified EFA6 apparently promotes efficient guanine nucleotide exchange exclusively on ARF6 (Chavrier and Franco, 2001; Macia et al., 2001; Franco et al., 1999).

Interestingly, the Sec7 domain has been suggested to be responsible for the specificity of small ARF-GEFs for class I ARF proteins under certain conditions. For
instance, the isolated ARNO Sec7 domain was shown to only be active on ARF1, but not on ARF6 (Franco et al., 1998). On the other hand, it is possible that the observed promiscuity in vitro of all the ARF-GEFs in general and of the small ARF-GEFs in particular is due to the highly conserved nature of the Sec7 domain. Table 1.5 summarizes reported distinct substrate specificities of all ARF-GEFs with respect to different ARF isoforms.

Furthermore, polyphosphoinositides have been shown to play a role in determining the specificity of the small GEFs for ARFs. As indicated earlier, it has been shown that cytohesin-1 was able to effectively catalyze guanine nucleotide exchange on ARF1 and ARF6 in vitro (Knorr et al., 2000). Interestingly, the addition of PIP3 strongly suppressed this exchange activity on ARF6 while enhancing it on ARF1. Thus, it appears that cytohesin-1 is capable of discriminating between ARF1 and ARF6 in vitro when the small ARF-GEF binds PIP3 indicating a possible functional role for this phosphoinositide in the regulation of the recruitment of ARF1 in vivo. However, there may be other functional events in the cell in which the small ARF-GEFs display selectivity for ARF6.

1.8.2.4. GEF binding partners: regulation by polyphosphoinositides and novel proteins

As already indicated, the binding of the GEFs via their PH domain to polyphosphoinositides such as PIP2 and PIP3 is crucial for their functions as this protein-phospholipid interaction dramatically enhances the rate of ARF nucleotide exchange by concentrating the GEFs on the bilayer membrane where the ARFs also localize through their myristoylated N-terminal α-helix. PIP2 was the first polyphosphoinositide found to be involved in the recruitment of ARNO and therefore
Table 1.5. Substrate specificities of all ARF-GEFs for the three classes of ARF isoforms and their subcellular localization and activity towards BFA

<table>
<thead>
<tr>
<th>ARF specificity</th>
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<th>Localization</th>
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<td>ARF 1</td>
<td>ARF 2</td>
<td>ARF 3</td>
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Large ARF-GEF family

| Gea1/2p | | | | | sensitive | Golgi |
| GBF1 | | | | | resistant | Golgi |
| Gnom/Emb30 | | | | | sensitive | ND |
| Sec7p | | | | | sensitive | Golgi |
| p200 | | | | | sensitive | Golgi |
| BIG1/BIG2 | | | | | sensitive | Golgi |

Intermediate ARF-GEF family

| ARF-GEF,100 | | | | | resistant | Cell periphery |

Small ARF-GEF family

| ARNO-1 (or cytohesin-2) | | | | | resistant | PM/Golgi |
| GRP-1 (or ARNO-3 or cytohesin-3) | | | | | resistant | PM/Golgi |
| cytohesin-1 (or B2-1 or ARNO-2) | | | | | resistant | PM/Golgi |
| cytohesin-4 | | | | | resistant | ND |
| EFA 6 | | | | | resistant | PM |

\(\checkmark/\checkmark/\checkmark\): preferred substrate in vitro. References are included in the text. PM, plasma membrane; ND, not determined.
the activation of ARF nucleotide exchange \textit{in vitro} (Chardin \textit{et al.}, 1996). Following
this discovery, several groups have shown a preference by ARNO (Venkateswarlu \textit{et al.}, 1998), GRP-1 (Klarlund \textit{et al.}, 1998) and cytohesin-1 (Nagel \textit{et al.}, 1998) PH domains for PIP_3 over PIP_2 \textit{in vitro}. This reported specificity of the GEFs for PIP_3 was supported by the fact that the transient recruitment of ARNO (Venkateswarlu \textit{et al.}, 1998), GRP-1 (Langille \textit{et al.}, 1999; Venkateswarlu \textit{et al.}, 1998) and cytohesin-1 (Nagel \textit{et al.}, 1998) to the plasma membrane was a response to agonists that activate the Class I PI-3-kinases, the source of PIP_3. This recruitment was in each case inhibited by PI 3-kinase inhibitors wortmannin and LY294002. In addition to biochemical studies, structural studies also favor the view that there is a stronger binding-affinity amongst ARF-GEF PH domains for PIP_3 over PIP_2 (Ferguson \textit{et al.}, 2000; Lietzke \textit{et al.}, 2000). However, since data presented in this thesis address this current view the rationale for this differential affinity will be considered in more detail elsewhere.

In addition to the important role of polyphosphoinositides in the regulation of the activity of the small ARF-GEFs, recent reports identified novel interacting proteins, such as IPCEF1 (Venkateswarlu, 2003), Cbyr (Tang \textit{et al.}, 2002), CASP (Mansour \textit{et al.}, 2002), GRSP1 (Klarlund \textit{et al.}, 2001a), GRASP also known as tamalin (Kitano \textit{et al.}, 2002; Nevrivy \textit{et al.}, 2000), and Munc13-1 (Neeb \textit{et al.}, 1999). These target proteins are believed to have the same role as the polyphosphoinositides in the GEF function \textit{i.e.} recruitment of the GEFs to membranes. Contrary to the situation with polyphosphoinositides, they interact with the coiled-coil domain of the small ARF-GEFs indicating a different aspect of protein targeting. The functional relevance of
these interactions have not been fully examined although Cbyr has been shown to enhance cytohesin-1 activity in vitro (Tang et al., 2002). More interesting still is the model of the small GEF-ARF interaction on the Golgi membrane proposed by Lee and Pohajdak (2000). In this model, the ARF-GEF uses its coiled-coil N-terminus to bind a membrane-bound adaptor protein. The authors of this work suggested that the coiled-coil N-terminus is in fact necessary and sufficient to target the small ARF-GEFs to the Golgi membrane, therefore making the PH domain functionally redundant. Alternatively, it is also likely that the coiled-coil-domain-dependent membrane targeting mechanism via direct association with an adaptor protein enhances or stabilizes the membrane interaction of the ARF-GEF with polyphosphoinositides through the PH domain. As a result, it is conceivable that this double interaction supports a favorable orientation of the ARF-GEF that would allow its Sec7 domain to activate more efficiently the GDP/GTP exchange on membrane associated ARF than in cases of single interaction.

Thus, in summary, the positive coordinated regulation of PLD by ARFs and their small exchange factors is likely to rely on their multiple interactions with PLD-controlling proteins (e.g. G-proteins, protein kinases) and polyphosphoinositides (PIP$_2$ and PIP$_3$).

1.8.3. Evidence for a coordinated regulation of PLD by ARF and its GEFs

Although the activation of PLD by ARFs is well-established in most in vitro systems, very little is known about the exact molecular mechanisms coupling the cell surface receptors to the ARF-dependent activation of PLD. Over the past few years, studies in intact and permeabilized cells have implicated ARF as a mediator of
receptor-stimulated PLD activity in response to agonists including FMLP, phorbol esters and growth factors in a number of cell lines (human neutrophils and neutrophil-related HL-60 cells, rat fibroblast cells, human embryonic kidney cells) (Li et al., 2003; Shome et al., 1998; Shome et al., 1997; Fensome et al., 1996; Rumenapp et al., 1995). A role for ARF in receptor-regulated PLD activity relies on the following findings: 1) BFA inhibited PDGF-, insulin- and PMA-induced PLD activation; 2) addition of recombinant ARF restores agonist-dependent PLD activation to permeabilized, cytosol-depleted cells; 3) dominant-negative ARF mutants (ARF1, ARF6) inhibited PLD stimulation by PDGF or PMA; 4) growth factor-promoted activation of ARF and recruitment to cellular membranes. Obviously, the observation that growth factors such as insulin and PDGF induced the activation of ARF, and its subsequent binding to cell membranes, suggests the involvement of ARF-GEFs in the signalling pathway controlling the activation of PLD. More recently, a study by Li et al. (2003) has implicated ARNO as a mediator of the activation of ARF1 and PLD by insulin in rat fibroblast cells in which both human insulin receptors and ARNO were overexpressed. In this study, insulin was found to promote the translocation of ARNO to cell membranes confirming earlier observations of insulin-dependent translocation of ARNO (Venkateswarlu et al., 1998) and GRP-1 (Langille et al., 1999) to the plasma membrane of murine 3T3 L1 adipocytes and Chinese hamster ovary (CHO-T), respectively. In addition, the translocation of cytohesin-1 from the cytosol to the plasma membrane in response to growth factor stimulation has also been demonstrated in PC12 cells (Venkateswarlu et al., 1999). The translocation of ARNO, GRP-1 and cytohesin-1 mentioned in those reports was blocked by wortmannin, suggesting the
participation of the PI 3-kinase pathway for the small ARF-GEFs recruitment to the plasma membrane of the studied cells. Interestingly, Li et al. (2003) showed that the recruitment of ARNO on to the plasma membrane was accompanied by activation and subcellular translocation of ARF1. Thus, these two events correlated well with the previously observed insulin-stimulated PLD activity in intact cells (Shome et al., 1997). These findings suggest an appealing model of a coordinated regulation of PLD by ARF and its exchange factor(s) involving the timely activation of the PI 3-kinase pathway in response to stimulation by insulin receptors (Li et al., 2003; Rizzo and Romero, 2002).

However, such a mechanism might be restricted to the rat fibroblast cells and the particular type of agonist used i.e. insulin. Therefore, the stimulation of different types of cell-surface receptors might trigger various signalling mechanisms in different cell types. It is difficult to confidently extrapolate a particular model of the mechanism of PLD activation from a single type of cell.

In order to clarify the role of the small ARF-GEFs in the ARF-regulated PLD activity, the main approach adopted in the work described in this thesis uses a cell permeabilization technique in which HL-60 cells, the selected cell line, are depleted of ARFs and other cytosolic components. The experiments proceed through the subsequent reconstitution of PLD activity by the reintroduction of recombinant ARFs and small ARF-GEFs in the presence of GTP. This system bypasses receptor-stimulation step and therefore allows the characterization the individual signalling components thought to participate in PLD regulation.
Of note, HL-60 cells have proven to be an exceptionally useful model system for the analysis of various aspects of the regulation of PLD for several reasons. First, they are of human origin and can be used, with certain limitations, as a model system for neutrophils and monocytes. Furthermore, HL-60 cells possess several membrane receptors, many of them G-protein coupled, such as FMLP receptors, PAF receptors, histamine H₁ and H₂-receptors, adenosine receptors (Gessi et al., 2002) and purinergic receptors (Adrian et al., 2000). Interestingly, the ARF-activated PLD isoform that is predominantly expressed in HL-60 cells is PLD1 (Colley et al., 1997). Moreover, previous reports indicate that BFA does not influence the stimulation of PLD activity by FMLP in HL-60 cells (Fensome et al., 1998; Bourgoin et al., 1995) or by ARF in Golgi-enriched membranes of HL-60 cells (Guillemain and Exton, 1997), suggesting both the presence and function of BFA-insensitive ARF-GEFs in human granulocytes. Specifically, cytohesin-1 has been identified in human neutrophils and HL-60 cells (Garceau et al., 2001). This supports a key role for cytohesin-1 and potentially its counterparts, ARNO and GRP-1, in agonist regulation of PLD in HL-60 cells.

Since cultured cell lines are often derived from patients suffering from cancer, and cancer cells have been shown to upregulate the PLD proteins, caution must be exercised when evaluating data from cell lines because they may not represent the situation in primary cells. This observation is important, because the majority of studies have utilised cultured cell lines for the analysis of PLD localization.

1.8.4. Role of the GAPs in the inactivation of ARFs

GAPs stimulate ARF-bound GTP hydrolysis and return ARF to the inactive GDP-bound state. Many new ARF GAP proteins have been identified and have been
categorized into three groups: Arf GAP1 type, Git type and AZAP type (Nie et al., 2003). These new ARF GAPs are multidomain proteins that were identified as binding partners of signal transduction molecules. All these proteins share a common GAP domain of 70 amino acids which include a zinc finger motif that is essential for GAP activity (Donaldson and Jackson, 2000). In addition to the zinc finger, all ARF GAPs have a conserved arginine within the GAP domain. Mutation of this arginine to lysine results in a 100,000-fold decrease in GTPase activity for ASAP1 (Randazzo et al., 2000) indicating that this arginine is essential for GAP activity.

Since ARF1 is involved in the formation of coatamer from Golgi vesicles, the disassembly of coatamer requires that the small GTP-ARF1 hydrolyzes its bound GTP by the action of a GAP. In vitro, the binding of the ARF1 GAP to lipid vesicles and its activity on membrane-bound ARF1-GTP are increased by diacylglycerols with monounsaturated acyl chains, such as those arising in vivo as secondary products from the hydrolysis of PC by ARF-activated PLD. Thus, the PLD pathway may provide a feedback mechanism that promotes GTP hydrolysis on ARF1 and the consequent uncoating of vesicles (Antonny et al., 1997).

It has been suggested that the ARF GAPs do more than turn off ARF. They provide the link between the ARF GTPase cycle and various signal transduction events in the cell. For instance, ASAP1 has been shown to coordinate cell signaling, the actin cytoskeleton, and the ARF nucleotide cycle. Overexpression of ASAP1 in cells results in a loss of focal adhesions and an inhibition of cell spreading and PDGF-induced ruffling. ASAP1 can work in vitro as a GAP on ARF1, ARF5, and ARF6, albeit more effectively on ARF1 and ARF5. These observations, together with the
recent indication that ARF6 affects cortical actin by activation of a polyphosphoinositide 4-phosphate 5-kinase (Honda et al., 1999), support a role for ARFs and ASAP1 in growth factor-stimulated reorganization of the actin cytoskeleton.

1.9. Principal aims of thesis

The major purpose of this thesis is to bring more insight into the complex regulation of PLD1 with respect to its numerous regulators. This thesis focuses on specific aspects of PLD1 regulation by taking advantage of the use and comparison of the cell-free and the permeabilized-cell model systems.

The first aim of this study is to establish a direct comparison between the effects of ARF1 and ARF6 on PLD1 activity in an attempt to rigorously establish if there is any selectivity for particular ARF isoforms in PLD1 activation.

The second aim will focus on the role of the small ARF-GEFs in regulating the activation of ARF proteins and their specific interactions with polyphosphoinositides in vitro. In particular, experiments will determine whether the reported specificity of certain small ARF-GEFs towards PIP3 is in fact a function of the specific interaction between the PH domains of these ARF-GEFs with this polyphosphoinositide. This is important since other agents like GEF-interacting proteins might also contribute to any effective discrimination of the ARF-GEFs for PIP3 and PIP2.

The third aim will be the study the effects of coupling the ARFs and their exchange factors and the consequences for PLD activity both in vitro and permeabilized HL-60 cells. In addition, polyphosphoinositides will be tested for their effects in this tripartite system.
Chapter two

Materials and Methods
2.1. Materials

2.1.1. Reagents

Culture medium RPMI-1640 and Medium 199 were purchased from Sigma. Supplements to these media are fetal calf serum obtained from Imperial Laboratories, penicillin (5000 units/mL) and streptomycin (5000 μg/mL) obtained from Invitrogen.

Culture medium IPL-41 for insect cells and supplements such as fungizone liquid (250 μg/mL), yeastolate ultrafiltrate (200 g/L) were purchased from Invitrogen. Other supplements to this medium are tryptose phosphate broth solution (29.5 g tryptose phosphate broth/L in deionised water) from Sigma and fetal calf serum.

Radiochemicals such as \([\text{methyl-}^3\text{H}]\) choline chloride (79 Ci/mmol) and 1,2-didecanoyl-sn-glycero-3-phospho[\text{H}]choline (85 Ci/mmol) were purchased from Amersham Pharmacia Biosciences; \([^{35}\text{S}]\)GTPγS (1250 Ci/mmol) was from NEN.

All other reagents including fatty acid-free bovine serum albumin, amberlite CG-50 (weakly acidic cation exchanger), ammonium sulfate, benzamidine, bromophenol blue, EGTA, HEPES, kanamycin, L-α dimyristoyl (C14) phosphatidylcholine, myristic acid, nalidixic acid, PE (from egg yolk), PIPES, PMSF, potassium chloride, sodium chloride, SDS, sodium phosphate, SLO, suramin, TEMED, Tris, and Triton X-100 were from Sigma. Ampicillin, ATP (disodium salt), GTP (lithium salt) and GTPγS were purchased from Roche. Calcium chloride solution, chloroform, EDTA (disodium salt), glycerol, glycine, hydrochloric acid, imidazole, IPTG, lysozyme, magnesium chloride, methanol, PBS, reduced glutathione, sodium azide, sodium hydroxyde were from BDH Laboratory supplies. APS, 40% Acrylamide/Bis solution and Supported Nitrocellulose membrane (0.2 μM) were from...
Bio-Rad. Coomassie Brilliant blue R250 was from Fluka, glutathione sepharose 4B from Amersham Biosciences, DTT from Alexis Biochemicals, hybond-C extra-membranes from Amersham Pharmacia Biotech, Ni-NTA from Qiagen, VectaSpin Micro 10 μM Polypropylene Mesh filters from Fisher, Ultima Gold scintillation liquid from Packard BioScience, tissue culture flasks from Helena BioSciences. Synthetic lipids such as 1,2-dibromostearoyl-sn-glycero-3-phosphocholine (PC) and dipalmitoyl phosphatidyl-L-serine (PS) and brain L-α-PIP₂ were obtained from Avanti Polar Lipids. The indicated phosphoinositides used in the liposome-binding assay and the protein-lipid overlay assay were purchased from CellSignals, Inc (Ohio, USA).

2.1.2. Preparation of 100 mM stock MgATP

A stock of 100 mM MgATP was prepared as follows: 1.815 g of di-sodium ATP was added to 15 mL of distilled water and stirred until the ATP was dissolved. Then 3 mL of 1 M MgCl₂ was added under constant stirring followed by the addition of 6 mL of 1 M Tris (pH > 10) to buffer the 2 moles/mole of protons accompanying the ATP. The pH of the solution was checked and adjusted to pH 7.0 with HCl. The volume was made up to 30 mL taking at least one wash from the beaker. The pH was checked a second time and readjusted to pH 7.0 if necessary. Aliquots of the solution were stored at -20°C.

2.1.3. Preparation of Ca²⁺ buffers

Two stock solutions were prepared. Firstly, EGTA solution: 100 mM EGTA, 20 mM Na⁺-PIPES pH 6.8, 73 mM MgCl₂. Secondly, Ca/EGTA solution: 100 mM Ca²⁺-EGTA 20 mM Na⁺-PIPES pH 6.8, 67 mM MgCl₂. Various ratios of these two solutions were mixed to give 100 mM stock solutions of calcium buffers. For example,
pCa7 stock buffer is prepared by mixing EGTA solution and Ca$^{2+}$-EGTA solution in the ratio 7.008:0.992 and pCa5 in ratio 0.496:7.504 respectively. Each solution gives about 2 mM free MgCl$_2$ final concentration when the buffer is diluted to 3 mM final EGTA concentration in the experiments described below.

2.1.4. Preparation of myristic acid and BSA

Myristic acid bound to BSA was prepared as follows: 1 g of BSA and 126 mg of myristic acid were dissolved in 25 mL of water in each of two separate tubes. The solution of myristic acid was warmed in a microwave as myristic acid is relatively insoluble in water at room temperature. The myristic acid solution was then mixed up with the BSA solution in a single tube (50 mL total volume). The newly prepared solution was kept in a water bath at 42°C prior to use.

2.1.5. Preparation of nalidixic acid

Nalidixic acid (25 mg/mL) was prepared as follows: 125 mg of nalidixic acid was added to 5 mL of water. A few microlitres of sodium hydroxyde were added to clarify the solution. The solution of nalidixic acid was prepared immediately before use.

2.1.6. Preparation of 6X SDS sample buffer

6X SDS sample buffer (0.376 M Tris, pH 6.8, 12% SDS, 60% (w/v) glycerol, 0.6 M DTT, 0.06% (w/v) bromophenol blue) was prepared as follows: 9.4 mL of 1 M Tris, pH 6.8 were diluted into 15.6 mL of water in a beaker. Then 15 g of glycerol (liquid), 3 g of SDS (solid), 15 mg of bromophenol blue and 2.3 g of DTT (solid) were added to the Tris buffer. The solids were allowed to dissolve. Another extra 15 g of
glycerol was finally added when the solution was all dissolved. Aliquots of 6X SDS sample buffer were stored at -20°C.

2.1.7. Sources of cell lines and viruses

HL-60 human promyelocytic leukemic cell line was purchased from the European Collection of Animal Cell Culture (UK).

Sf9 (Spodoptera frugiperda) insect cells were a kind gift from Dr Claudia Wiedemann. The recombinant baculovirus encoding for the hPLD1b was kindly provided by Prof. Michael J.O. Wakelam (Birmingham University, UK).

2.2. Methods

2.2.1. Cell culture and radiochemical labelling

2.2.1.1. Promyelocytic HL-60 cells

HL-60 cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, penicillin (50 units/mL) and streptomycin (50 µg/mL). Cells were grown to a density of approximately $1 \times 10^6$ cells/mL in a humidified atmosphere containing 5% CO$_2$ at 37°C.

For experiments, cells were cultured in Medium 199 supplemented with 10% (v/v) dialyzed heat-inactivated fetal calf serum, penicillin (50 units/mL) and streptomycin (50 µg/mL) and grown in the presence of $[^{3}H]$Choline (1 µCi/mL) for 48 hours.

2.2.1.2. Sf9 cells

The Sf9 cells were grown in monolayers at 27°C in tissue culture flasks, using IPL-40 insect medium supplemented with 10% (v/v) fetal calf serum, 1% (v/v) amphotericin B (Fungizone), 8% (v/v) tryptose phosphate broth solution and 2% (v/v)
yeastolate ultrafiltrate. For the production of recombinant hPLD1b, the cells were
grown in a monolayer up to $5 \times 10^7$ cells per $175$ cm$^2$ culture flask in $20$ mL IPL-40
insect medium with supplements except for fungizone. An inoculum of the
recombinant baculovirus encoding hPLD1b was added to the cell culture at a
multiplicity of infection of around $100$ pfu/cell.

For recombinant virus amplification, new insect cells were infected with the
recombinant baculovirus and were incubated for 7 days at $27^\circ$C in IPL-40 insect
medium containing all of the mentioned supplements. The cells were dislodged from
the surface by gentle scraping and then centrifuged at $1000 \times g$ for $10$ min at $4^\circ$C and
the resulting supernatant containing the new stock of virus was passed through
$0.22 \mu M$ Millex filter (Millipore) and kept at $4^\circ$C in the dark for short-term storage.

2.2.2. Construction of the ARF expression plasmid pMon 5840-ARF1 (His)$_6$ and
5840-ARF6 (His)$_6$

DNA coding for ARF proteins was amplified by PCR from authentic samples
using forward and reverse primers that coded for the termini of the sequence and any
desired extensions. These extensions comprised 5' sequence for an Nco-I restriction
site and 3' sequence containing codons for six consecutive histidine residues followed
by a Hind III restriction site. For example, the following primers were used for bovine
ARF1:

N-terminus: (sense strand):

$$5' - \text{TATATACC\cdot ATG\cdot GGG\cdot AAT\cdot ATC\cdot TTT\cdot GCA\cdot AAC\cdot CTC}-3'$$
C-terminus: (antisense strand):

\[5' - \text{ATTATTAAGCTTCA} \cdot \text{GTG} \cdot \text{ATG} \cdot \text{ATG} \cdot \text{ATG} \cdot \text{ATG} \cdot \text{ATG} \cdot \text{TTT} \cdot \text{CTG} \cdot \text{GTT} \cdot \]
\[\text{CCG} \cdot \text{GAG} \cdot \text{CTG} \cdot \text{ATT} \cdot \text{GGA} \cdot \text{CAG}-3'\]

Restriction sites are underlined, ARF1 sequence and reverse compliment ARF1 sequence are shown in red, the stop codon is in bold and the 6xHis coding sequence is in blue. Related primers, but with sequence corresponding to fragments of ARF1 or ARF6, were designed along similar lines with all restriction sites and other features as for the ARF1 primers. PCR products were digested with NcoI and HindIII, purified and ligated into a sample of plasmid pMon5840 (see below) which had been previously cut with the same two restriction enzymes. After transformation into \textit{E.coli} XL1 Blue cells and selection by ampicillin resistance, plasmid DNA was isolated from positive clones and the insert sequenced to exclude any mutations. Plasmids were then transformed into \textit{E.coli} BL21-DE3 pLysS cells with or without co-transformation with expression plasmid pBB131 coding for the yeast \textit{N}-myristoyltransferase 1 (NMT1 and see below) and selected by ampicillin or ampicillin plus kanamycin resistance respectively. Positive or double positive clones were expanded and stored as frozen glycerol stocks until required.

2.2.3. pBB131 (yeast NMT1) plasmid

The expression plasmids pMon5840 and pBB131 were a kind gift of Dr J.I. Gordon (Washington University Medical School, Missouri, USA).
2.2.4. Expression of proteins in *E. coli* and subsequent purification of 6xHis-tagged recombinant ARFs fusion proteins

Recombinant non-myristoylated and myristoylated ARF1, ARF6 and [Δ17]ARF1 (ARF1 with amino acids 1-17 deleted) were prepared as described previously (Randazzo *et al.*, 1995) with a few modifications.

The preparation of [Δ17]ARF1 (His)_6 did not require the co-translational addition of myristic acid as it is an ARF1 mutant in which the N-myristoylation site is deleted.

For the expression and purification of ARF proteins the BL21 (DE3) *E. coli* bacteria transformed with either pMon5840-ARF1 (His)_6 or pMon5840-ARF6 (His)_6 each with or without pBB131 (yeast NMT1) plasmids were used. NMT catalyses the *in vivo* transfer of a myristate group to a glycine residue at position 2 of the ARF protein. Bacteria lack this type of transferase activity, hence its coexpression with the different plasmids coding for ARF proteins if myristoylation is required. A 6xHis tag was inserted into the sequence for ARF1, ARF6 and [Δ17]ARF1 at the C-terminus as described above. Since the pMon5840 plasmids carry ampicillin resistance and pBB131 carries kanamycin resistance all cultures of the double or singly transformed bacteria can be selected at all points by combinations of these antibiotics e.g. ampicillin (100 μg/mL) and kanamycin (50 μg/mL).

An initial culture of transformed cells were grown in 5 mL of LB (Invitrogen) overnight at 37°C in presence of the appropriate antibiotic. Cells from the overnight culture were expanded in two conical flasks containing 200 mL of LB, once again containing antibiotics, and allowed to grow overnight at 37°C. The next day the cells
were expanded in four 1-litre flasks of LB for 1 hour. A freshly prepared solution containing myristic acid (2.52 mg/mL) and BSA (0.02 g/mL) was then added to the growing cells (12.5 mL in each flask) for an extra hour. The temperature was then reduced to 26°C to optimize the yield of myristoylated proteins as previously shown by Franco et al. (1995). Cells were left for a further hour to cool down to the new temperature. Where required the expression of NMT was then induced with 1 mM IPTG. Nalidixic acid (25 mg/mL) was added to the cells (at the same time as the IPTG if required) to induce ARF protein expression (1.25 mL of the freshly prepared nalidixic acid stock solution was added to each flask). After 3 hours of protein expression, further myristic acid and BSA were added and the culture was then allowed to grow overnight at 26°C. The cells were harvested by centrifugation at 4420 × g for 10 min at 4°C using a JA-10 fixed angle rotor in a J2-21 Centrifuge (Beckman). The cell pellet from a 4-litre culture was then resuspended in a total volume of 80 mL of lysis buffer 1 (50 mM Tris, pH 8.0, 25% (w/v) sucrose, 0.02% (w/v) NaN₃, 1 mg/mL lysozyme) and incubated with gentle stirring for 30 min at room temperature. The cells were lysed by the addition of 32 mL of lysis buffer 2 (50 mM Tris, pH 8.0, 100 mM MgCl₂, 0.2% (w/v) Triton X-100, 0.02% (w/v) NaN₃). In addition, 1 mM DTT and PMSF were added to the suspension which was then incubated for 30 min at 4°C with stirring. The cells were subsequently homogenized by using a Citenco homogenizer (type KQTS7). The homogenate was clarified by ultracentrifugation at 100,000 × g for 60 min at 4°C using a Type 35 Beckman rotor in a Sorvall Ultracentrifuge (OTD 65B model). ARF proteins were then purified from the supernatant through two steps. Firstly, the supernatant obtained after
ultracentrifugation was pumped through a 25 mL bed volume column of Ni-NTA fast-flow beads with a Minipuls II pump (Gibson) at about 2 mL/min. The column was previously equilibrated with the following cycle: 100 mL of Nickel column buffer containing 20 mM NaH$_2$PO$_4$, 20 mM Tris, 300 mM NaCl, 0.2% (w/v) NaN$_3$, pH 6.0, followed by 100 mL of the same buffer supplemented with 0.5 M imidazole and then followed by 50 mL of a mixture (5:2 ratio) of lysis buffers 1 and 2 respectively. The Ni-NTA column was then connected to an FPLC system which was set at a flow rate of 2.5 mL/min. After a washing step, the protein was eluted with a continuous gradient of 0 to 0.5 M imidazole in the Nickel column buffer. This particular column chromatography step was performed at room temperature or at 4°C with identical results. Fractions of 5 mL were collected. In a second chromatographic step, myristoylated ARFs were resolved from the underivitised forms by hydrophobic interaction chromatography on phenyl superose if required. The pooled samples from the Ni-NTA column were mixed with solid ammonium sulphate to a final concentration of 1 M. In the cold, the pH was adjusted to 7.6 and the sample clarified by centrifugation and passage through a 0.45 μM filter. The HR10/10 phenyl Superose column was equilibrated in a cold Tris buffer (20 mM Tris, pH 7.6 and 0.02% (w/v) NaN$_3$) containing 1 M (NH$_4$)$_2$SO$_4$. After passage of the sample through the column the bed was washed with one volume of buffer containing 1.7 M (NH$_4$)$_2$SO$_4$. The column was then developed with a decreasing linear ammonium sulphate gradient in the same Tris buffer and 8 mL fractions were collected. All flow rates were 0.5 mL/minute or less. Protein elution was monitored by absorbance at 280 nm. Protein-containing fractions were dialysed against several changes of Tris buffer (100:1 volume ratios as

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above) in the cold and then concentrated as described for other samples and stored frozen in small aliquots. Alternatively, the myristoylated protein could be collected by differential ammonium sulphate precipitation. Peak fractions from the Ni-NTA chromatography step containing the ARF protein mixture were pooled and dialyzed against 1.7 M (NH₄)₂SO₄, pH 7.6 overnight at 4°C. The resultant precipitate containing myristoylated ARF proteins was collected by centrifugation at 27000 × g using a JA-20 fixed angle rotor (Beckman) for 15 min at 4°C. The pellet containing the protein sample was dissolved in 20 mM Tris, pH 7.6 and subsequently dialyzed three times against the same buffer at a volume about 100 times the volume of the protein sample to remove the highly concentrated salt (see “Protein dialysis” section and following sections for more details on protein analyses).

We could not detect biochemical difference between forms of myristoylated ARFs prepared by either of these two methods.

The extent of myristoylation of the protein preparations was assessed by reverse phase HPLC as myristoylated forms of the proteins show a characteristic increase in retention time.

2.2.5. Construction of the recombinant plasmids pQE30 containing the coding sequences for full-length ARNO, GRP-1 and cytohesin-1

A sample of the pQE30 expression plasmid with an insert coding for human cytohesin-1 was the generous gift of Dr. Joel Moss, NHLBI, Bethesda, Maryland, USA. cDNAs coding for other ARF-GEF proteins were amplified by PCR using forward and reverse primers that coded for the termini of the sequence and any desired extensions. Authentic DNA coding for GRP-1 was from Dr. J. Klarlund, University of
Massachusetts Medical Center, Massachusetts, USA. DNA coding for ARNO was obtained by direct PCR from a mouse foetal brain cDNA library kindly provided by Professor C. Carpenter, Harvard Medical School, Boston, USA. In both cases the extensions comprised 5' sequence for a KpnI restriction site and 3' sequence for a Hind III restriction site. For example, the following primers were used for mouse GRP-1:

N-terminus (sense strand):

5' - ATTGGTACC GAC GAA GGC GGT GGC GGT GAG GGC GGC - 3'

C-terminus: (antisense strand):

5' - TAAAAGCTT CTA TTT CTT ATT GGC AAT CCT CCT TTT - 3'

Restriction sites are underlined, GRP-1 sequence and reverse compliment GRP-1 sequence are shown in red and the stop codon is in bold. Subsequent cloning into pQE30 then gives DNA coding for an N-terminal 6xHis extension to the ARF-GEF translation products. Related primers, but with complimentary and reverse complimentary sequence corresponding to ARNO-1, were designed along similar lines with all restriction sites and other features as for the GRP-1 primers. PCR products were digested with KpnI and Hind III, purified and ligated into a sample of plasmid pQE30 (see below) which had been previously cut with the same two restriction enzymes. After transformation into E.coli XL1 Blue cells and selection by ampicillin resistance, plasmid DNA was isolated from positive clones and the insert sequenced to exclude any mutations. Plasmids were then transformed into E.coli M15[pREP4] cells and again selected by ampicillin resistance. Colonies of positive clones were expanded in suspension culture and samples frozen as glycerol stocks until required.
2.2.5.1. Recombinant full-length ARNO-1 (His)_6, GRP-1 (His)_6 and cytohesin-1 (His)_6

*E. coli* strain M15[pREP4] harboring the recombinant pQE30 plasmids containing in frame coding sequences for full-length ARNO, GRP-1 and cytohesin-1 were used for 6xHis-tagged recombinant protein expression. Consequently, the protein products could be purified by Ni-NTA chromatography using exactly the same procedure as described above for the purification of ARF proteins.

2.2.5.2. Recombinant ARNO, GRP-1 and cytohesin-1 GST-Cys-tagged PH domains

The ARF-GEF PH domains were kindly provided by Gerald Hammond (Cancer Research UK, London). Briefly, the recombinant pGEX-4T3 plasmids containing the coding sequences for PH domains of ARNO (residue 235 to the C-terminus), GRP-1 (residue 240 to the C-terminus) and cytohesin-1 (residue 236 to the C-terminus) were transformed individually into the *E. coli* strain TG1. By this cloning procedure a GST-Cys fusion tag was inserted into each protein at the N-terminus of the mentioned PH domains. This allowed expression and purification of the PH domains by standard methods for GST-tagged fusion proteins. Briefly, after cell lysis and ultracentrifugation, the GST-PH domain proteins present in the supernatant were incubated with glutathione sepharose 4B beads and rotated at 4°C overnight. After washing away unbound proteins the fusion proteins were then eluted with 10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0.

2.2.6. Expression of GST-hPLD1b in sf9 insect cells and its subsequent purification

A baculovirus-based expression system was used to obtain recombinant N-terminus-GST-tagged hPLD1b. Sf9 insect cells infected with the baculovirus encoding
the hPLD1b were harvested after 48 hours of infection. The cells were centrifuged for 10 min at 1000 \times g at 4°C and were washed in cold PBS. The cells were then resuspended in ice-cold lysis buffer (20 mM Tris, 1 mM EGTA, 1 mM MgCl₂, 0.1 mM DTT, 0.1 mM PMSF, 0.1 mM benzamidine, pH 7.5) and disrupted by brief sonication. The resultant suspension was centrifuged at 1500 \times g for 10 min at 4°C and the supernatant recovered. The supernatant was then centrifuged at 16000 \times g for 30 min at 4°C to pellet the cellular debris. The clarified supernatant from this second centrifugation was mixed with 1 mL of packed glutathione sepharose 4B beads and rotated at 4°C overnight. The beads were subsequently washed 3 times with 10 mL lysis buffer and the newly purified GST-hPLD1b captured on beads was stored at 4°C (without elution) in the same buffer. On average, the activity of the PLD1b used in separate incubation in each experiment (20 μL PLD 50% slurry beads) was 7 fmol/min PC-hydrolyzed.

2.2.7. General protein handling and quality control procedures: protein dialysis, protein concentration, protein quantification, protein storage, protein electrophoresis, protein staining and destaining, gel drying and western blotting

2.2.7.1. Protein dialysis to remove imidazole and ammonium sulphate

After the elution step at the end of the purification process, the protein sample to be dialyzed was transferred into a soaked dialysis tubing 12 kDa molecular weight cut-off (Sigma) which was then carefully clamped and placed into a 5 L container filled with 20 mM Tris, pH 7.6 at a volume about 100 times the volume of the protein sample. The container was then placed in a cold room set at 4°C. To facilitate the removal of high salts, a spin bar was placed in the bottom of the container. The protein
sample was dialyzed for 24 hours with gentle stirring. Changes of buffer were performed 2 more times giving a one million-fold theoretical salt dilution.

2.2.7.2. Protein concentration

After dialysis, the purified protein was transferred to a properly preassembled 50 mL capacity Amicon concentrator (Millipore) containing a 10,000 MW-cut off Amicon ultrafiltration membrane (YM10 Millipore). The ultrafiltration membrane was placed in the bottom of the concentrator with its glossy side up. A nitrogen gas head at an operating pressure read between 20 psi (1.4 kg/cm²) and 30 psi (2.1 kg/cm²) was applied to the concentrator during stirring.

Subsequently, a further concentration of protein sample was also performed using 10 kDa MW-cut off Microsep microconcentrators (Pall, Gelman Laboratory) in order to get a smaller volume of protein sample (< 0.5 mL). A maximum of 3.5 mL of protein sample was transferred into the sample reservoir of the microconcentrator which was then centrifuged at 3000 × g using a JA-20 fixed angle rotor (Beckman) for 30 min at 4°C.

2.2.7.3. Protein quantification

The concentrated protein sample (except for hPLD1b which was isolated on glutathione sepharose beads) was quantified by using a dye-binding assay based on the method of Bradford (Bio-Rad or Pierce kit). The amount of protein in the sample was measured according to the manufacturer’s instructions. The protein samples were read using an Ultrospec II spectrophotometer (LKB Biochrom) with reference to a BSA standard curve. Usually, final unmyristoylated and myristoylated ARF concentrations ranged from 2.5 mg/mL to 3 mg/mL. Concentrations between 5 mg/mL to 12 mg/mL
were usually obtained for the GEF recombinant proteins ARNO, cytohesin-1 and GRP-1.

2.2.7.4. Protein storage

All recombinant proteins were dated and stored at -20°C for short-term storage and -80°C for long-term storage.

2.2.7.5. Protein electrophoresis

Protein samples were first separated by SDS-PAGE. The following protocol was used to analyze the ARF and GEF recombinant proteins purified as described above. A 1 mm thick, 12.5% SDS-PAGE separating gel was made with a discontinuous stacking gel (≤ 5%). The separating or lower gel was made first by mixing 10 mL of separating gel buffer (1.5 M Tris, pH 8.8 with HCl, 0.4% (w/v) SDS) with 17.5 mL of water, 12.5 mL of 40% acrylamide/bis solution, 400 μl of 20% (w/v) APS and 30 μL TEMED. The separating gel was then poured between the slabs of the preassembled SE 600 vertical slab gel unit (Hoefer) in the dual gel casting stand to a level about 4 cm from the top of the gel. A few microlitres of water-saturated isobutanol were added on top of the separating gel to eliminate any air bubbles and prevent oxygen diffusion. While polymerization of the separating gel was taking place, the stacking or upper gel was prepared by mixing 5 mL of stacking buffer (0.5 M Tris, pH 6.8 with HCl, 0.4% (w/v) SDS), 12.7 mL water, 2 mL of 40% acrylamide/bis acrylamide solution, 100 μL of 20% (w/v) APS and 10 μL TEMED. After polymerization of the separating gel, the small excess of alcohol was poured off and the surface of the polymerized separating gel before it was washed 3 times with distilled water. The stacking gel was then poured in on top of the separating gel. A 1 mm thick, 20-well
A gel comb was subsequently inserted, taking care not to trap any air bubbles below the teeth of the comb. The gel was allowed to sit for at least 30 min. After polymerization, the comb was gently removed from the stacking gel and the newly formed wells were filled with tank buffer (0.025 M Tris, 0.192 M glycine, 0.1% SDS, pH 8.3).

The protein samples were diluted in a total volume of 50 μL to get approximately 5 μg of protein per tube to which 10 μL of 6X SDS sample buffer were added. The samples were heated to about 80°C for 5 min and then loaded in the wells.

The SE 600 vertical slab gel unit was then connected to an Electrophoresis Power supply (Model 3000 Xi, Bio-Rad) and the gel run for 14 hours at 9 mA constant current per gel.

2.2.7.6. Protein staining and destaining

A portion or all of the SDS-PAGE gel was placed in a staining solution (0.025% Coomassie Brilliant blue R 250 (Fluka), 40% (v/v) methanol, 7% (v/v) acetic acid) for periods between approximately 3 hours and overnight under gentle shaking.

In order to reveal the separated protein bands, the staining solution was replaced by a destaining solution (40% (v/v) methanol, 7% (v/v) acetic acid). Changes of the destaining solution were performed several times until the gel background became clear. Finally the destained gel was washed in water.

In some cases, the staining and destaining steps could be accelerated by repeated heating of the staining and destaining solutions using a standard microwave.

The destained gel was either stored in water at 4°C or dried under vacuum.
2.2.7.7. Gel drying

Vacuum drying was applied to the destained gel. The gel was placed on a piece of wet filter paper (Whatmann number 1) which was then laid onto the plate of a gel dryer (Model 583, Bio-Rad). The top of the gel was covered with a piece of Saran plastic wrap. The silicon dryer cover flap was then lowered onto the gel, the vacuum turned on to seal the flap. The gel was usually dried at 70°C for 1 hour and 30 min.

2.2.7.8. Western blotting

Western blotting was performed for the recombinant ARF proteins using the following protocol: one portion of the SDS-PAGE gel containing resolved protein bands following electrophoresis was cut off and washed 2 times in transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) MeOH) for 15 min each time. A similarly equilibrated semi-dry transfer system was applied for the protein gel in a Multiphor II Electrophoresis System (Pharmacia Biotech). For each gel, one sheet of 0.2 μM pore nitrocellulose filter membrane (Bio-Rad) and 12 sheets filter paper (Whatman) were cut the same size as the gel and soaked in transfer buffer. Prior to protein transfer, a transfer sandwich was assembled on the anode plate in the following order: 6 sheets of filter paper, nitrocellulose membrane, SDS-PAGE gel, 6 sheets of filter paper. The Multiphor II Electrophoresis System was connected to a power supply (2303 Multidrive XL, LKB Bromma). The protein sample was transferred to the nitrocellulose membrane for 1 hour using 0.8 mA/cm² current density. After protein transfer, the nitrocellulose membrane was incubated in blocking solution containing 5% (w/v) skimmed milk powder in 100 mL TBS Tween buffer (100 mM Tris/HCl, 0.9% NaCl, w/v 0.1% (w/v) Tween-20) for 1 hour at room temperature on a lab shaker
(gently rocking). After blocking, the membrane was washed 3 times in 100 mL TBS Tween for 10 min each time. ARF protein was then detected by incubating the membrane with a mouse monoclonal 1D9 antibody (Alexis) used at 1:2000 dilution in TBS-Tween buffer for 1 hour at room temperature. After 3 buffer washes in TBS Tween, the membrane was subsequently incubated with secondary antibody goat anti mouse HRP (horseradish peroxidase) conjugate (Bio-Rad) at 1:3000 dilution in 100 mL TBS Tween containing 5% (w/v) skimmed milk powder and 1% goat serum for 1 h at room temperature. The membrane was then washed 3 times in TBS Tween for 10 min each time and subsequently incubated with 10 mL of an HRP substrate solution made up of one volume of Stable Peroxide Solution and one volume of Luminol/Enhancer Solution (West Pico Chemiluminescent substrate, Pierce) for 10 min at room temperature. Following the incubation time with chemiluminescent reagents, the membrane was laid flat between sheets of clear plastic and developed by short exposure in the FujiFilm Intelligent Dark Box. Images were captured and stored electronically.

2.2.8. PLD assay in SLO-permeabilised HL-60 cells

Because the major goal of this research is to understand the intracellular mechanisms that lead to the activation of PLD, it has been necessary to gain access to the interior of the cell. To achieve this HL-60 cells were permeabilized with the pore-forming-toxin SLO. SLO is a cytolytic protein secreted by the bacterial genus *Streptococcus* (Bhakdi et al., 1985). This toxin specifically binds to cholesterol in the plasma membrane and forms arc- and ring-shaped oligomers that penetrate the apolar domains of the plasma membrane. The ring-shaped structures once embedded in the
plasma membrane of the cells form transmembrane channels of between 25 and 30 nm in diameter. At the concentrations used in this study SLO generally preserves the architectural integrity of the cells. Thus the formation of these pores results in the loss of cytosolic components (except for those that have strong interactions with membranes or the cytoskeleton) and enables the introduction into the cells of specific recombinant proteins, nucleotides, and other low molecular weight agents that influence the activity of PLD.

Different types of assays have been reported for the determination of PLD activity. In our laboratory, we use a headgroup-release assay which measures the formation of \([methyl-^3H]\)choline from \([methyl-^3H]\)choline-labeled PC. At the end of the radiolabelling period, the cells were centrifuged and washed in the assay buffer (137 mM NaCl, 2.7 mM KCl, 20 mM PIPES, 1 mg/mL BSA, 0.02% NaN₃, pH 6.8). The resuspended cells were then treated with SLO (0.4 IU/mL final concentration) and EGTA (0.2 mM). EGTA was included in the buffer to chelate any free calcium released during permeabilisation. The cells were permeabilised for 10 min at 37°C. Proteins such as ARF proteins were shown to leak out of the cells within 5-10 min (Fensome et al., 1996). At the end of the permeabilisation step, the permeabilised cells were sedimented by centrifugation at 2000 × g for 5 min to remove the cytosolic components that had leaked out during permeabilisation. The permeable HL-60 cells were then resuspended in buffer containing 3 mM MgATP and 3 mM Ca²⁺/EGTA buffer (pCa5). Reaction mixtures (50 μL final volume) comprising 20 μL of permeabilised cells and permuted combinations of recombinant ARFs, GEFs, Arfaptin proteins, with or without the non-hydrolyzable GTP-analogue GTPγS in 30 μL were
carried out for 20 min at 37°C. All mixtures were made on ice and the reactions started by transferring the test tubes to a 37°C water bath. The incubation was terminated by addition of 500 µL methanol/chloroform (1:1, v/v). Phases were separated by addition of 250 µL water followed by vigorous mixing and centrifugation. [3H]Choline was recovered from the upper aqueous phase by cation-exchange chromatography as follows: 400 µL of the upper aqueous phase of each reaction was loaded into the cation-exchange resin (Amberlite CG-50) columns. The columns were rinsed with water to elute the phosphorylated choline metabolites. [3H]Choline was then eluted directly into scintillation vials with 1 mL of elution buffer (50 mM glycine in 500 mM NaCl, pH 3.0), mixed with 5 mL Ultima Gold scintillation cocktail and quantified by liquid scintillation analysis.

2.2.9. Activity of GST-hPLD1b on glutathione sepharose beads quantified in vitro

The recombinant human PLD1b activity was assayed by measuring the free choline released upon phosphatidylcholine hydrolysis. Immediately before each experiment substrate was prepared from a mixture of PE, PIP2, and didecanoyl (including some radiolabelled material) PC in the molar ratio 10:0.3:1, with a final concentration of PC at 8.6 µM exactly as described by Vinggaard et al. (1996). [3H]Choline-labelled didecanoyl PC was added to give approximately 200000 d.p.m. per 10 µL of substrate. The lipid mixture was “dried down” to a glass under a stream of N2 and resuspended in the substrate buffer. The substrate and the assay buffers were made up in 50 mM Na/Hepes (pH 7.5), 3 mM EGTA, 80 mM KCl and 1 mM DTT, with the addition of 0.5 mM MgCl2 and 2 mM CaCl2 in the assay buffer. Vesicle formation was carried out by a combination of hard vortexing and sonication (3 × 20
sec on a medium setting). Assays were then carried out by mixing combinations of the following reaction components on ice: 20 μL GST-hPLD1b on glutathione sepharose beads (50% slurry approximately), 10 μL substrate, 30 μL combinations of ARFs, GEFs, Arfaptins, C₈ polyphosphoinositide lipids, nucleotides in a final volume of 60 μL. The exact concentrations are indicated in the legends of the corresponding figures presented in subsequent chapters. The reaction mixtures were transferred to a 37°C water bath and incubated for 1 hour.

2.2.10. Nucleotide binding assay

The assay is based on the stimulation of [³⁵S]GTPγS binding to ARP proteins, the exchange reaction being either spontaneous or catalyzed by a GEF. The assay was performed as follows: a recombinant ARF protein was diluted to a final concentration of 1 μM in an exchange buffer containing 50 mM Hepes, pH 7.5, 1 mM MgCl₂, 100 mM KCl, 1 mM DTT, 12 μM [³⁵S]GTPγS and 1 mM phospholipid vesicles in 50 μL volume of reaction. Where present GEFs were at a final concentration of 1 μM. Phospholipids vesicles used in the exchange assays were prepared as follows: solutions of L-α dimyristoyl (C14) PC in chloroform and PIP₂ in chloroform:methanol:H₂O (1:1:1, v/v) were mixed in the required proportion (50:1, molar concentration). The mixture was dried under nitrogen and resuspended in exchange buffer. The suspension was vortexed thoroughly and sonicated until the solution clarified.

Reaction mixtures were incubated at 37°C for between 5 and 10 min depending on the type of recombinant ARF proteins used. All of the exchange reactions are linear over this period. The reactions were stopped by transferring the samples to an ice
water bath followed by the addition of 150 μL ice-cold Triton X-100 solution (w/v 1.67%). The ARF proteins along with any bound [35S]GTPγS were recovered by immediately mixing the reactions with nickel-coated agarose beads for 10 min at 4°C to recover the polyhistidine-tagged ARFs. Specifically, 50 μL beads (20% slurry in exchange buffer) were added to the reaction mixture and placed on a rotary mixer. Samples of beads were collected by brief centrifugation on 10 μM pore size Polypropylene Mesh filters and the beads washed extensively in washing buffer (20 mM NaH2H3PO4, 20 mM Tris, 300 mM NaCl, 0.02% w/v NaN3, pH 6.0) to remove unbound [35S]GTPγS. ARFs were then specifically eluted from the nickel beads, with 60 μL elution buffer (20 mM NaH2H3PO4, 20 mM Tris, 300 mM NaCl, 0.02% w/v NaN3, 0.5 M imidazole, pH 6.0) and the amount of eluted [35S]GTPγS estimated by scintillation counting. It was established in preliminary experiments there is little or no further detectable exchange of GTPγS onto ARFs under the reaction stop conditions, even in the presence of GEFs. Hence the assay faithfully reports only the exchange catalysed in the initial incubation before quenching.

2.2.11. Protein-liposome-binding assay

Mixtures of PC and PS (4:1 molar ratio) with and without the indicated phosphoinositides (3 Mol% of total lipids) in methanol:chloroform (1:1 v/v) containing 0.4% (w/v) 1 M HCl were dried under N2. The lipid mixtures were resuspended in a buffer containing 10 mM Tris-HCl, pH 7.5, and 75 mM NaCl at a total lipid concentration of 25 mM. The lipid mixtures were maintained at 65°C and regularly vortexed. After 1 hour of rehydration, the mixtures were alternately frozen in dry-ice and thawed five times by transferring to the heat block for 5 min each time.
Unilamellar liposomes were then formed by multiple passages (> 30) through a Mini-
Extruder (Avanti Polar Lipids) equipped with a 0.1 μm pore polycarbonate membrane
(Whatman). Purified GST-Cys-PH domains (10 μM total protein) and full-length
6xHis-tagged ARF guanine nucleotide exchange factors (5 μM total protein) were
incubated with liposomes (2 mM total lipid) for 1 hour at room temperature in buffer
containing 50 mM Tris-HCl, pH 8.3, 75 mM NaCl, 1 mM EDTA, 2 mM DTT in a total
volume of 100 μL. The liposomes were pelleted by ultracentrifugation at 25000 x g
for 1 hour at 25°C. The amount of protein that remained in the supernatant was
quantified using a dye-binding assay based on the method of Bradford (Bio-Rad) and
compared with that of a control reaction that contained proteins without lipids.

2.2.12. Protein-lipid overlay assay

A protein–lipid overlay assay was performed using either full-length
6xHis-tagged GEFs and their respective isolated GST-Cys-tagged PH domains. Lipid
solution (1 μL) containing between 6.25 and 200 pmoles of phosphoinositides
dissolved in chloroform:methanol:water (1:2:0.8, v/v) was spotted onto Hybond-C
extra-membranes and dried at room temperature for 1 hour. The membrane was
blocked in 3% (w/v) fat-free BSA in TNE (50 mM Tris (pH 7.5), 150 mM NaCl,
1 mM EDTA) that contained 0.1% Tween-20 at 4 °C overnight. Membranes were
incubated at room temperature with the same solution containing 1μg/mL of the
indicated GEF proteins for 1 hour, washed five times in TNE containing 0.1% (v/v)
Tween-20, and then incubated for 1 hour with a mouse monoclonal anti-penta-his
antibody used at 1:1000 (Qiagen) or with an anti-GST antibody (1:1000 dilution).
After washing, the membranes were incubated with goat anti-mouse HRP-conjugated
antiserum at 1:2500 (Pierce) or with HRP-conjugated goat anti-rabbit antibody (Boehringer Mannheim; 1:2500 dilution) for 1 hour at room temperature. The membranes were washed four times at room temperature in TNE containing 0.1% (v/v) Tween 20, once with Tris-buffered saline (20 mM Tris (pH 7.5), 150 mM NaCl), and signals were detected by enhanced chemiluminescence (NEN Life Science Products).

2.2.13. Expression of data

All results presented are from individual experiments repeated at least three times with similar results. Within each of these individual experiments all individual measurements were made as duplicates or occasionally triplicates. Data and statistical analysis were performed by SigmaPlot 8.0 and Excel softwares. Data are shown as mean ± SEM unless otherwise stated. Statistical significance of the values was evaluated by Student's t-test. Oftentimes it is necessary to describe the significance of statistical relationships between various different measurements within one experiment. Under these circumstances precise P values are described in the accompanying text while code letters indicate significance in the diagrams.

In Chapter 3, EC$_{50}$ and V$_{max}$ values in some experiments were determined by non-linear regression curve fitting of concentration-response data fitted to the equation $f(x) = (m + M - m)/(1 + (x/EC_{50})^h)$, where m and M are the minimal and maximal responses, respectively, x is the ARF protein concentration, and h is the Hill slope (SigmaPlot 8.0).
The analyses of the amino acid sequences of the proteins of interest described in this thesis were conducted as described in the legends of the corresponding figures and tables.

Further analysis was performed to determine the extent of sequence identity between representative members of a particular protein.
ARF isoform specificity of PLD studied in permeabilized HL-60 cells and a cell-free system
3.1. Introduction

As indicated in Chapter one, six mammalian ARF family members have been identified and they have been classified into three categories based on their size and sequence similarity homology. ARF1, ARF2, and ARF3 form class I, ARF4 and ARF5 constitute class II, and ARF6 represents class III (Moss and Vaughan, 1995). Unlike other GTP-binding proteins, ARFs are myristoylated on an N-terminal glycine in a cotranslational event catalyzed by N-myristoyltransferase (NMT) (Kahn et al., 1988). This lipid modification plays a critical part in ARF function including the activation of PLD. All classes of ARFs have been shown to activate PLD. However, it remains a point of contention as to whether there is any difference in their potency. Early studies showed little difference (Brown et al., 1995; Massenburg et al., 1994) but some later work showed significant difference (Jones et al., 1999; Sung et al., 1999). In an attempt to redress the comparative lack of side-by-side studies of ARF isoforms in PLD activation, the principal aim of this chapter is to decide if ARF1 and ARF6 have distinct or similar effects on PLD activity. Using a bacterial expression system based on the yeast myristoyl-CoA: protein NMT coexpression system of Duronio et al. (1990), recombinant fully and partially myristoylated ARF1 and ARF6, non-myristoylated ARF1 and ARF6 and deletion mutant human [Δ17]ARF1 were synthesized in E. coli, purified to homogeneity and characterised in order to give a set of comparable reagents to investigate their specific role in the activation of PLD. Well-established cell permeabilization and a new cell-free system were used to carry out these studies.
3.2. Results

3.2.1. Purification of ARF proteins

ARF proteins purified from bacteria were subjected to two consecutive purification steps. ARF proteins obtained from cell lysis were first loaded on Ni-NTA column and eluted with a linear gradient of imidazole (Figure 3.1). A single symmetric peak for each ARF isoform was obtained and the corresponding fractions under this peak were pooled and loaded on a phenyl Superose column in a second chromatographic step in which myristoylated ARFs were resolved from the underivitised forms by hydrophobic interaction chromatography. Example of a chromatogram of phenyl Superose fractionation step in ARF1 purification is shown in Figure 3.2. The peaks of myristoylated ARF1 and nonmyristoylated ARF1 are indicated.

The extent of myristoylation of the protein preparations was determined by reverse phase HPLC. The myristolated forms of the proteins showed a characteristic increase in retention time confirming earlier HPLC analyses of purified recombinant ARF proteins (Randazzo and Kahn, 1995). The profile of myristoylated and nonmyristoylated ARF1 and ARF6 is shown in Figures 3.3 and 3.4.

SDS-PAGE analysis indicated a 21 kDa band corresponding to the expected molecular weight of ARF1 (Figure 3.5, upper panel). In addition, Western blot analysis confirmed the identity of the ARF protein as the antibody 1D9 reacted strongly with ARF1 (Figure 3.5, lower panel). Of note, the mobility of myristoylated ARF1 on SDS-PAGE was greater than that of non-myristoylated ARF1. This result confirms an earlier observation by Franco et al. (1995).
Figure 3.1. Elution of ARF proteins from Ni-NTA column. The supernatant obtained after cell lysis was applied to a 25 mL bed volume column of Ni-NTA, which had previously been equilibrated in 100 mL of Nickel column buffer (20 mM NaH₂PO₄, 20 mM Tris, 300 mM NaCl, 0.2% NaN₃ (w/v), pH 6.0) followed by 100 mL of the same buffer supplemented with 0.5 M imidazole and then followed by 50 mL of a mixture (5:2 ratio) of lysis buffers 1 and 2 respectively (see Materials and Methods section for more details). Proteins were eluted with a linear gradient of 0 to 0.5 M imidazole in the Nickel column buffer. Fractions of 5 mL are collected. Protein elution was monitored by absorbance at 280 nm.
Figure 3.2. Elution of myristoylated ARF1 from phenyl-Superose. Fractions pooled from the Ni-NTA column were concentrated and made to 1 M with solid ammonium sulphate and applied to the phenyl-Superose column equilibrated with buffer containing 1 M ammonium sulphate. Proteins were eluted with a descending ammonium sulphate gradient and 8 mL fractions were collected. Protein elution was monitored by absorbance at 280 nm. The peaks of nonmyristoylated (peak 1) and myristoylated (peak 2) are indicated.
Figure 3.3. Analysis by HPLC of purified bovine ARF1. Bovine ARF1 was coexpressed with NMT and purified as described in Materials and Methods section. (A) The two peaks correspond to nonmyristoylated ARF1 ($R_T \approx 23$ min) and myristoylated ARF1 ($R_T \approx 34$ min). (B) The two peaks correspond to myristoylated ARF1 ($R_T \approx 28$ min) and 100% myristoylated ARF1 ($R_T \approx 41$ min). The two separate experiments illustrate two points. Firstly that the absolute retention times can vary from experiment to experiment. Secondly a sample of myristoylated ARF1 without resolution on phenyl Superose shows two peaks of protein in chromatogram (A) while the peaks can be seen to have been resolved in the two separate, but overlaid, chromatograms in (B).
Figure 3.4. Analysis by HPLC of purified mouse ARF6. Mouse ARF6 was coexpressed with NMT and purified as described in Materials and Methods section. (A) The second peak corresponds to nonmyristoylated ARF6 ($R_T \approx 15$ min). (B) The second peak corresponds to myristoylated ARF6 ($R_T \approx 26$ min).
Figure 3.5. *SDS-PAGE and Western blot analyses of purified myristoylated ARF1.* (Upper panel) SDS-PAGE analysis of fractions of myristoylated ARF1 following the hydrophobic interaction chromatographic step. The proteins were visualized with Coomassie blue dye. (Lower panel) Following transfer to nitrocellulose, blots were probed with an ARF-specific antibody (monoclonal antibody 1D9). Western blotting procedures are as described in Materials and Methods section.
3.2.2. ARF myristoylation enhances the activation of PLD in permeabilized HL-60 cells

In the following experiments the fraction of the total ARF1 protein derivatised with myristate was varied; either 0%, 35% or 100%. The percentage myristoylation of ARF6 was either 0% or > 90%. The 35% partial myristoylation of ARF1 is useful as this is the usual upper bound of derivatisation that seems to be achieved in prokaryotic expression systems by other investigators, although on the whole the average is probably between 10 to 20%.

Figures 3.6 and 3.7 show the comparison of partially myristoylated ARF1 (mARF1) and non-myristoylated ARF1 (ARF1) and partially myristoylated ARF6 (mARF6) and non-myristoylated ARF6 (ARF6) in their respective activation of PLD in permeabilized HL-60 cells. As illustrated in Figure 3.6, little PLD activity was generated by the 35% myristoylated ARF1 alone. However, the addition of GTP\(\gamma\)S greatly enhanced the 35%-myristoylated-ARF1-stimulated PLD activity. Its effect was concentration-dependent and was characterized by an EC\(_{50}\) value of 1.13 ± 0.54 \(\mu\)M and a predicted V\(_{\text{max}}\) of 2.71 ± 0.39\% of total choline-labelled lipids hydrolyzed (PLD activity). By contrast, the non-myristoylated ARF1 showed very little effect on PLD activity and the addition of GTP\(\gamma\)S barely improved its effect. An estimate of the activated non-myristoylated ARF1 in terms of potency was determined by noting that one has to titrate in about 30 times more of the non-myristoylated ARF1 to equal the PLD response elicited by for instance 0.20 \(\mu\)M 35% myristoylated ARF1 as indicated in Figure 3.6.
Figure 3.6. Effects of partially and unmyristoylated ARF1 proteins on PLD activity in permeabilized HL-60 cells. After 10 min permeabilization with SLO (0.4 IU/mL) at 37°C, [3H]choline-labelled and cytosol-depleted HL-60 cells were centrifuged and resuspended in buffer containing 3 mM MgATP and 3 mM Ca²⁺/EGTA (pCa5). The cells were then incubated for 20 min at 37°C with the indicated concentrations of recombinant partially and unmyristoylated ARF1 proteins in the presence or absence of 10 μM GTPyS. Reactions were stopped by transferring cells to ice and by adding methanol/chloroform (1:1, v/v). Phases were partitioned by addition of water. Release of [3H]choline was measured as described in the Materials and Methods section. The result represent one experiment conducted in duplicate. Inset: the data of the concentration-response relation for 35% myristoylated ARF1 plus GTPyS were fitted by nonlinear regression to a rectangular hyperbola with the use of SigmaPlot 8.0 software. The degree of curve fitting was indicated by the coefficient of correlation (R), which has the highest value of 1, when 100% fitting would be established. The concentration that gives 50% of maximum PLD response (EC₅₀) to 35% myristoylated ARF1 plus GTPyS was 1.13 ± 0.54 μM and the V₅₀ was 2.71 ± 0.39% total lipids hydrolyzed (PLD activity).
The requirement for myristoylation was also observed in experiments using the 90% myristoylated ARF6 and non-myristoylated ARF6. The results shown in Figure 3.7 firstly show that both myristoylated ARF6 and non-myristoylated ARF6 could activate PLD activity without the requirement for a guanine nucleotide in a concentration-dependent fashion. The myristoylated form displayed a significantly higher potency than its non-myristoylated counterpart at concentrations > 100 nM (P < 0.05, n = 3) except at 0.9 μM ARF6 where the myristoylated and non-myristoylated proteins did not show any significant difference in activating PLD (P = 0.07, n = 3). The subsequent addition of GTPγS to myristoylated ARF6 increased PLD activity. This increase in PLD response to myristoylated ARF6 plus GTPγS was significant at concentrations ranging from 0 to 0.3 μM myristoylated ARF6 (P < 0.05, n = 3). However, the observed enhancement of PLD activity was mostly due to the presence of GTPγS rather than the protein. Similar effects on PLD were observed for the non-myristoylated GTPγS-activated ARF6. Not too surprisingly, the myristoylated GTPγS-activated ARF6 was significantly more efficient in activating PLD than its non-myristoylated GTPγS-activated counterpart at concentrations between 0.03 μM and 0.09 μM ARF (P < 0.05, n = 3). At concentrations above 0.09 μM ARF6, no significant difference between the stimulatory effects on PLD activity elicited by the GTPγS-activated myristoylated and non-myristoylated ARF6 proteins was detected (P > 0.05, n = 3). In addition, the PLD response induced by the 90% myristoylated ARF6 in the presence of GTPγS reached a plateau at concentrations > 100 nM and significantly decreased at 10 μM myristoylated ARF6 when compared with the PLD response to 0.09 μM myristoylated ARF6 (P = 0.03, n = 3). This decrease in PLD
Figure 3.7. Effects of partially and unmyristoylated ARF6 proteins on PLD activity in permeabilized HL-60 cells. After 10 min permeabilization with streptolysin O (0.4 IU/mL) at 37°C, [³H]choline-labelled and cytosol-depleted HL-60 cells were centrifuged and resuspended in buffer containing 3 mM MgATP and 3 mM Ca²⁺/EGTA (pCa5). The cells were then incubated for 20 min at 37°C with the indicated concentrations of recombinant partially and unmyristoylated ARF6 proteins in the presence or absence of 10 μM GTPγS. Reactions were stopped by transferring cells to ice and by adding methanol/chloroform (1:1, v/v). Phases were partitioned by addition of water. Release of [³H]choline was measured as described in the Materials and Methods section. Data shown are mean ± S.E.M. of duplicate samples from 3 experiments. The significance of data was evaluated using Student's t.
activity might be due to the activation of inhibitory pathways by high concentrations
of myristoylated GTPγS-activated ARF6. Alternatively, direct inhibition of PLD
activity may as well occur.

Taken together, the results described so far confirmed previous studies on the
critical role of myristoylation in the ARF-dependent PLD activity (Massenburg et al.,
1994; Brown et al., 1993).

3.2.3. Comparison of the stimulation of PLD activity by myristoylated ARF1 and
ARF6 in permeabilised HL-60 cells and cell-free experiments

After examination of the literature on ARF1 and ARF6 effects on PLD activity, it
emerges that this thesis is the first to present a direct comparison between the effects
of properly characterised, myristoylated ARF1 and ARF6 on PLD activity in both cell-
based and cell-free systems.

In the permeabilized HL-60 cell model system, 35% myristoylated ARF1 and
90% myristoylated ARF6, the highest percentage of myristoylation available at the
time of the assays for each of these proteins, were tested alongside each other. The
results are illustrated in Figure 3.8. The activity of PLD was equally stimulated by
35% myristoylated ARF1 and 90% myristoylated ARF6 in a concentration-dependent
way at concentrations ranging from 0 to 0.9 μM ARF proteins in the presence and in
the absence of GTPγS. However, both ARF isoforms strongly relied on the presence
of GTPγS for optimal PLD activation. The basal activity of PLD was significantly
augmented by such concentrations of 35% myristoylated ARF1 alone as 0.3 μM (P =
0.03, n = 4), 0.9 μM (P = 0.01, n = 4), 2.9 μM (P = 0.01, n = 4) and 9 μM (P = 0.002,
n = 4). The addition of GTPγS to 35% myristoylated ARF1 significantly further
Figure 3.8. Activation of PLD by myristoylated ARF1 and ARF6 in permeabilized HL-60 cells. After 10 min permeabilization with SLO (0.4 IU/mL) at 37°C, $[^3]$H]choline-labelled and cytosol-depleted HL-60 cells were centrifuged and resuspended in buffer containing 3 mM MgATP and 3 mM Ca$^{2+}$/EGTA (pCa5). The cells were then incubated for 20 min at 37°C with increasing concentrations of recombinant ARF1 and ARF6 in the presence or absence of 10 μM GTPyS. Reactions were stopped by transferring cells to ice and by adding methanol/chloroform (1:1, v/v). Phases were partitioned by addition of water. Release of $[^3]$H]choline was measured as described in the Materials and Methods section. Data shown are mean ± S.E.M. of duplicate samples from 4 experiments. The significance of data was evaluated using Student's t. Inset: the data of the concentration-response relation for 35% myristoylated ARF1 plus GTPyS were fitted by nonlinear regression to a rectangular hyperbola with the use of SigmaPlot 8.0 software. The degree of curve fitting was indicated by the coefficient of correlation (R), which has the highest value of 1, when 100% fitting would be established. The concentration that gives 50% of maximum PLD response (EC$_{50}$) to 35% myristoylated ARF1 plus GTPyS was 1.56 ± 0.20 μM and the V$_{max}$ was 1.27 ± 0.05% total lipids hydrolyzed (PLD activity).
potentiated its activation of PLD over the whole range of selected concentrations (P < 0.05, n = 4). Fitting a rectangular hyperbola concentration-response curve to the data obtained for 35% myristoylated ARF1 plus GTPγS gave an EC\textsubscript{50} value of 1.56 ± 0.20 μM and a V\textsubscript{max} value of 1.27 ± 0.05% total choline lipids hydrolyzed (PLD activity). Similar to the effects of 35% myristoylated ARF1 alone, the basal PLD activity was significantly increased by such concentrations of 90% myristoylated ARF6 alone as 0.08 μM (P = 0.02, n = 4), 0.3 μM (P = 0.01, n = 4), 0.9 μM (P = 0.02, n = 4), 2.9 μM (P = 0.04, n = 4) and 9 μM (P = 0.02, n = 4). Although the activation of PLD mediated by 35% myristoylated ARF1 seemed to be slightly better than 90% myristoylated ARF6 at 9 μM ARF proteins in the absence of GTPγS, this difference was however not significant (P = 0.19, n = 4). Adding GTPγS to 90% myristoylated ARF6 significantly improved its stimulatory effects on PLD activity at concentrations ranging from 0 to 2.9 μM ARF proteins (P < 0.05, n = 4). However, as previously observed in Figure 3.7 this increase in PLD activity mediated by myristoylated ARF6 was principally due to the effects of GTPγS. Notably, 90% myristoylated ARF6 plus GTPγS apparently reduced PLD activity at 9 μM ARF protein but this decrease was not significant in comparison to the PLD response induced by 2.9 μM 90% myristoylated ARF6 plus GTPγS (P = 0.10, n = 4). Interestingly, this apparent inhibitory effect on PLD activity induced by high concentrations of myristoylated GTPγS–activated ARF6 was previously observed in Figure 3.7.

Thus, the effects of both myristoylated ARF1 and ARF6 on PLD activity in the presence of GTPγS were not significantly different at concentrations of ARF up to 2.9 μM (P > 0.05, n = 4). However, at the highest concentration of ARF proteins used,
that is 9 μM, myristoylated ARF1 was significantly and unequivocally more efficient in activating PLD than myristoylated ARF6 (P = 0.02, n = 4). In summary, at high concentrations 35% myristoylated ARF1 achieved a stronger activation of PLD than 90% derivitised ARF6 in the presence of GTPγS. This prompts two plausible working hypotheses. Firstly, ARF1 at very high percentage myristoylation may prove to be an even stronger activator compared to ARF6 in HL-60 cells. Alternatively, higher myristoylation may reveal an inhibitory component to ARF1 action at high protein concentrations. These two hypotheses are tested below and the results reported later in this chapter.

In order to determine whether such an apparent discrepancy between ARF1 and ARF6 potency can also be seen *in vitro*, two fully myristoylated ARF1 and ARF6 were employed to activate a recombinant human PLD1b isoform expressed in sf9 cells, partially purified and immobilised on beads. The use of PLD1b isoform in the cell-free system described in this thesis was prompted by *in vitro* studies which previously demonstrated that only PLD1 isoforms including PLD1a and PLD1b were ARF-dependent (Powner *et al.*, 2002; Hodgkin *et al.*, 1999; Hammond *et al.*, 1997). Besides, PLD1 isoforms are thought to be the forms of PLD activated by ARF1 in permeabilized HL-60 cells (Gibbs and Meier, 2000; Marcil *et al.*, 1997; Ohguchi *et al.*, 1997; Saqib and Wakelam, 1997). As illustrated in Figure 3.9, myristoylated ARF1 and ARF6 had no effect on recombinant PLD1b activity in the absence of GTPγS. However, the addition of GTPγS significantly enhanced the stimulatory effects on PLD1b exerted by myristoylated ARF1 and to some extent by ARF6 in comparison with the PLD basal activity (P < 0.05, n = 4). Similar to their effects in
Figure 3.9. In vitro activation of recombinant human PLD1b by myristoylated ARF1 and ARF6. Sf9 cells were infected with recombinant baculovirus for the expression of hPLD1b which was recovered on glutathione sepharose beads. A sample of the hPLD1b on beads (20 µl) was incubated with the indicated proteins concentrations at 37°C for 60 min in the presence of PC substrate (8.6 µM final concentration) and [3H]Choline-labelled PC (200000 dpm per 10 µl substrate), with or without 10 µM GTPγS. Reactions were stopped by transferring cells to ice and by adding methanol/chloroform (1:1, v/v). Phases were partitioned by addition of water. Release of [3H]choline was measured as described in the Materials and Methods section. Data shown are mean ± S.E.M. of duplicate samples from 4 experiments. The significance of data was evaluated using Student’s t. Note: 100% PLD activation is equivalent to 4.45 ± 2.22% PC hydrolyzed. Inset: the data of the concentration-response relation for myristoylated ARF1 plus GTPγS were fitted by nonlinear regression to a rectangular hyperbola with the use of SigmaPlot 8.0 software. The degree of curve fitting was indicated by the coefficient of correlation (R), which has the highest value of 1, when 100% fitting would be established. The concentration that gives 50% of maximum PLD response (EC50) to myristoylated ARF1 plus GTPγS was 0.22 ± 0.06 µM and the Vmax was 99.12 ± 5.01% PLD activation.
permeabilized cells, both myristoylated, GTPγS-activated ARF1 and ARF6 did not show any significant difference in their ability to activate recombinant PLD1b at concentrations ranging from 0.02 μM to 0.6 μM ARF protein (P > 0.05, n = 4). The activation of PLD1b by myristoylated, GTPγS-activated ARF1 was clearly concentration-dependent and was characterized by an EC50 of 0.22 ± 0.06 μM and a Vmax of 99.12 ± 5.01% PLD activation. By contrast, and also similar to the effect of myristoylated ARF6 already observed in the permeabilized cell system shown in Figure 3.8, the activation of recombinant PLD1b by myristoylated ARF6 showed a biphasic trend since at concentrations above 1 μM ARF6 plus GTPγS became inhibitory. Indeed, 18.3 μM myristoylated, GTPγS-activated ARF6 significantly reduced the maximal PLD1b activation obtained at 0.6 μM by a factor of two (P = 0.04, n = 4).

Thus, the comparison between ARF1 and ARF6 in the two described model systems established parallels to some extent but above all clearly underlined critical differences between these two proteins in their regulation of PLD activity.

3.2.4. Activation of PLD requires the N-terminus of ARF but the deletion of the amino-terminal 17 residues from ARF does not affect the ability of ARF to bind guanine nucleotides with high affinity

Comparison between the 35% myristoylated ARF1, and a mutant form of ARF1, the [Δ17]ARF1, in which both the myristoylation site and an N-terminal α-helix with a key function are deleted by mutagenesis, is shown in Figure 3.10. [Δ17]ARF1 completely failed to activate PLD both at high concentration and in the presence of GTPγS compared with 35% myristoylated ARF1. Clearly these results demonstrate
Figure 3.10. Activation of PLD by myristoylated ARF1 but not by [Δ17]ARF1 (ARF1 with amino acids 1-17 deleted) in permeabilized HL-60 cells. After 10 min permeabilization with SLO (0.4 IU/mL) at 37°C, [3H]choline-labelled and cytosol-depleted HL-60 cells were centrifuged and resuspended in buffer containing 3 mM MgATP and 3 mM Ca\(^{2+}\)/EGTA (pCa5). The cells were then incubated for 20 min at 37°C with increasing concentrations of recombinant ARF1 and [Δ17]ARF1 in the presence or absence of 10 μM GTP\(_\gamma\)S. Reactions were stopped by transferring cells to ice and by adding methanol/chloroform (1:1, v/v). Phases were partitioned by addition of water. Release of [3H]choline was then measured as described in the Materials and Methods section. Data shown are mean ± S.E.M. of duplicate samples from 4 experiments. The significance of data was evaluated using Student's t. Inset: the data of the concentration-response relation for 35% myristoylated ARF1 plus GTP\(_\gamma\)S were fitted by nonlinear regression to a rectangular hyperbola with the use of SigmaPlot 8.0 software. The degree of curve fitting was indicated by the coefficient of correlation (R), which has the highest value of 1, when 100% fitting would be established. The concentration that gives 50% of maximum PLD response (EC\(_{50}\)) to 35% myristoylated ARF1 plus GTP\(_\gamma\)S was 1.56 ± 0.20 μM and the V\(_{\text{max}}\) was 1.27 ± 0.05% total lipids hydrolyzed (PLD activity).
that the activation of PLD in permeabilised HL-60 cells absolutely requires the N-terminus of ARF. This observation is in agreement with previous studies (Jones et al., 1999; Zhang et al., 1995).

However, the deletion of the N-terminus of ARF1 did not abolish its ability to bind guanine nucleotide, the binding of which is known to be catalysed by small GEFs such as ARNO, GRP-1 and cytohesin-1 (Jackson and Casanova, 2000). As shown in Figure 3.11, the deletion mutant was found to be capable of spontaneously binding \[^{35}\text{S}]\text{GTP\gamma S}\. The spontaneous guanine nucleotide binding was significantly increased by ARNO (\(P = 0.02, n = 3\)), GRP-1 (\(P = 0.05, n = 3\)) and cytohesin-1 (\(P = 0.03, n = 3\)). These GEFs were equally efficient in increasing the binding of \[^{35}\text{S}]\text{GTP\gamma S}\ to \[\Delta 17\]ARF1 as their exchange activities were not significantly different (\(P > 0.05, n = 3\)). Indeed, the guanine nucleotide exchange on \[\Delta 17\]ARF1 mediated by ARNO was not significantly different from that mediated by GRP-1 (\(P = 0.10, n = 3\)) and cytohesin-1 (\(P = 0.75, n = 3\)). Similarly, there was no significant difference between the exchange activity displayed by GRP-1 and cytohesin-1 (\(P = 0.11, n = 3\)).

In summary, these results suggest that the failure of \[\Delta 17\]ARF1 to activate PLD is not due to an inability to bind GTP\gamma S and subsequently undergo structural transitions in switch regions associated with this change.

3.2.5. Increasing the extent of myristoylation on ARF1 significantly enhanced its stimulatory effects on PLD activity

Figure 3.12 presents the concentration-response curves for the myristoylated forms of ARF1 in the absence and in the presence of GTP\gamma S and their effects on PLD activity in permeabilized HL-60 cells. In the case of 35% myristoylated ARF1, the use
Figure 3.11. GTPγS binding to [Δ17]ARF1 is increased by GEFs. Specific binding of [35S]GTPγS to [Δ17]ARF1 (1 μM) was determined after 1 min incubation at 37°C in exchange buffer containing 50 mM Hepes, pH 7.5, 1 mM MgCl₂, 100 mM KCl, 1 mM DTT, 12 μM [35S]GTPγS and 1 mM phospholipid vesicles in 50 μl volume of reaction where the present GEFs were at a final concentration of 1 μM. Data are means ± S.E.M., n=3 experiments. Asterisks indicate significantly different from the control (P < 0.05).
of the protein in the absence of GTPγS significantly increased the basal PLD activity at such concentrations as 3 μM (P < 0.01, n = 4) and 9.5 μM (P < 0.03, n = 4). Interestingly, increasing the degree of myristoylation to 100% substantially increased the potency of the protein (left shift, Figure 3.12). This is indeed supported by the fact that as little as 300 nM 100% myristoylated ARF1 alone significantly enhanced the basal PLD activity (P < 0.01, n = 4). Moreover, PLD activity elicited by 100% myristoylated ARF1 alone was significantly higher than that produced by 35% myristoylated ARF1 alone at concentrations > 100 nM ARF (P < 0.05, n = 4). Kinetic parameters were obtained by fitting a rectangular hyperbola concentration-response curve to the data for the effects of 100% myristoylated ARF1 in the absence of GTPγS. As a result, an EC\(_{50}\) value of 0.42 ± 0.04 μM and a V\(_{\text{max}}\) value of 0.42 ± 0.01% total choline lipids hydrolyzed confirmed the efficiency of 100% myristoylated ARF1 in stimulating PLD activity which was achieved in the absence of GTPγS.

Interestingly, the addition of GTPγS to both 35% and 100% myristoylated ARF1 resulted again in the shift of the potency of the proteins to the left but also increased the catalytic activity of PLD to a greater extent. Parameters obtained by the fitting procedure for 100% myristoylated ARF1 (in the absence of GTPγS: EC\(_{50}\) = 0.42 ± 0.04 μM and V\(_{\text{max}}\) = 0.42 ± 0.01% total choline lipids hydrolyzed; in the presence of GTPγS: EC\(_{50}\) = 0.05 ± 0.01 μM and V\(_{\text{max}}\) = 0.53 ± 0.02% total choline lipids hydrolyzed;) revealed the potentiating effect of GTPγS. As seen, GTPγS decreased the EC\(_{50}\) by a factor of 8 and increased the V\(_{\text{max}}\) by a factor of 1. Strikingly, these data show that GTPγS increases the sensitivity of PLD to ARF.
Figure 3.12. Effects of fully and partially myristoylated ARF1 proteins on PLD activity in permeabilized HL-60 cells. PLD activity was determined under standard assay conditions as described in Materials and Methods section. Data shown are mean ± S.E.M. of duplicate samples from 4 experiments. The significance of data was evaluated using Student’s *t*. Inset, right: the data of the concentration-response relation for the mentioned proteins were fitted by nonlinear regression to a rectangular hyperbola with the use of SigmaPlot 8.0 software. The degree of curve fitting was indicated by the coefficient of correlation (R), which has the highest value of 1, when 100% fitting would be established. The EC\textsubscript{50} of 100% myristoylated ARF1, 100% myristoylated ARF1 plus GTP\textgamma S, and 35% myristoylated ARF1 plus GTP\textgamma S were 0.42 ± 0.04 µM, 0.05 ± 0.01 µM and 0.24 ± 0.04 µM, respectively. The corresponding V\textsubscript{max} values were 0.42 ± 0.01% total lipids hydrolyzed (PLD activity), 0.53 ± 0.02% total lipids hydrolyzed (PLD activity), and 0.53 ± 0.02% total lipids hydrolyzed (PLD activity), respectively.
More intriguingly still is the combined effects of GTP\(\gamma\)S and myristoylation. Indeed, the 100% myristoylated GTP\(\gamma\)S-activated ARF1 was again a more potent activator of PLD than its 35% myristoylated, GTP\(\gamma\)S-activated counterpart. This is corroborated by the difference between the EC\(_{50}\) (0.05 \(\mu\)M) of 100% myristoylated GTP\(\gamma\)S-activated ARF1 and the EC\(_{50}\) (0.24 \(\mu\)M) of 35% myristoylated GTP\(\gamma\)S-activated ARF1. Thus increasing the percentage of myristoylation on ARF decreased its EC\(_{50}\) by a factor of 5. This result indicates an enhancement of ARF potency as a result of a better degree of myristoylation and the complementary effect of GTP\(\gamma\)S. Interestingly, both 100% myristoylated GTP\(\gamma\)S-activated ARF1 and 35% myristoylated GTP\(\gamma\)S-activated ARF1 shared the same V\(_{\text{max}}\) (0.53 ± 0.02% total lipids hydrolyzed). This result again clearly suggests that the maximal PLD activation strongly depends on the combined action of myristoylation and GTP\(\gamma\)S. Therefore, a legitimate question arises about the extent of the role of GTP and myristoylation in the ARF-mediated activation of PLD.

Closer analysis of the data shown in Figure 1.12 revealed that myristoylation in reality accounts for most of the change in affinity of ARF1 for PLD. Indeed, the average value found for the basal PLD activity was about 0.1% total choline lipids hydrolyzed. As already mentioned above, the V\(_{\text{max}}\) value achievable with 100% myristoylated ARF1 in the absence and in the presence of GTP\(\gamma\)S were 0.42 ± 0.01% and 0.53 ± 0.02% total choline lipids hydrolyzed, respectively. In view of these results, it emerges that myristoylation contributes to 75% of the overall potency of ARF1 in PLD activation, while the apparent effect of GTP\(\gamma\)S constitutes an additional 25% of the total PLD activation. This important observation prompts another critical
question about the mechanism(s) by which GTPγS contributes to the optimal activation of PLD by ARF1. It is possible that GTPγS effectively enhances the catalytic activity of PLD by increasing the sensitivity of ARF to PLD. For example, GTPγS may activate other G-proteins such as the Rho family small GTP-binding proteins and the membrane-associated heterotrimeric G proteins. These GTPγS-activated G-proteins, or their downstream effectors, would thereby synergize with ARF to bring about a full, maximal activation of PLD.

The observed increased activation of PLD by a fully myristoylated ARF1 could be due to a better nucleotide exchange on ARF1. This aspect of the ARF activity will be thoroughly considered in the next chapter.
3.3. Discussion

The fully and partially myristoylated and non-myristoylated recombinant ARFs were assayed for reconstitution of GTPγS-stimulated PLD activity in permeabilized HL-60 cells and in cell-free assays. The activation of PLD by ARF1 proteins in both model systems is concentration-dependent but also relies on the presence of guanine nucleotides (GTPγS in this work) for a full and optimal activation of the enzyme. The myristoylated forms of ARF proteins were found to be greatly more effective than its non-myristoylated counterpart in activating PLD confirming earlier findings (Tsai et al., 1998; Brown et al., 1995; Massenburg et al., 1994; Brown et al., 1993). The diminished potency of the non-myristoylated ARF proteins may be attributed to a lower affinity for cellular proteins, possibly the PLD enzyme or decreased nucleotide exchange efficiency. Therefore, one unifying hypothesis is that the non-myristoylated ARF proteins display a reduced membrane association since both interaction with PLD and exchange factors require interaction with membranes. This is a conventional interpretation: myristoylation is thought to greatly enhance the membrane association of the activated ARF since membrane targeting is conventionally thought to be a necessary step in the sequence of events leading to a full and effective activation of PLD and other ARF effectors such as vesicle coat proteins (Donaldson et al., 1992) and adaptors (Ooi et al., 1998; Stamnes and Rothman, 1993). The fact that ARF interacts with the vesicle coat proteins suggests that myristoylation must be involved in ARF-mediating events such as intra-Golgi (Palmer et al., 1993; Taylor et al., 1992) or endoplasmic reticulum to Golgi transport (Balch et al., 1992) and in endosome-endosome fusion (Lenhard et al., 1992).
Interestingly, the data presented in this chapter highlight for the first time the crucial fact that fully myristoylated ARF proteins or high concentrations of partially myristoylated ARF proteins can significantly achieve a potent activation of PLD without the requirement for guanine nucleotide. This interesting finding allows interpretation and reconciliation of the observation made by Powner et al. (2002). In a study investigating the in vivo regulation of PLD1b by Rho, ARF and PKC family proteins in antigen-stimulated RBL-2H3 cells, the authors demonstrated that these regulatory proteins bound directly and independently to PLD1b by using surface plasmon resonance (SPR) techniques. These results were further strengthened by co-localization studies that clearly showed that in antigen-stimulated RBL-2H3 co-transfected with PLD1b and its regulators, PLD1b co-localized with Rac1, ARF6 and PKCα predominantly within actin-rich structures. Interestingly, they claimed that some of their unpublished data showed that the interaction between ARF proteins and PLD1b was surprisingly independent of the guanine nucleotide binding also detected by SPR. However, the same authors had previously found that in vitro activation of PLD1b was dependent on the guanine nucleotide binding (Hodgkin et al., 1999). PLD activation is the result of a direct physical interaction between ARF and PLD as shown by in vitro PLD assay results described in this chapter and elsewhere (Powner et al., 2002; Hammond et al., 1997). It is possible that the high concentrations of ARF used in the broken HL-60 cells bring about sufficient transfer of protein to the intracellular membrane where PLD is localized and where the majority of PLD activity has been detected (Hodgkin et al., 1999) purely by mass action. This would increase the probability of the two proteins colliding and provide a purely mass-action-based
model of PLD activation. Therefore it is tempting to speculate that on the whole the activation of PLD could be triggered by a collision-governed mechanism. This mechanism would be independent of the presence of guanine nucleotide and therefore independent of the rearrangements of ARF switch 1 and 2 regions induced upon nucleotide exchange. Since the highly concentrated ARF proteins are fully myristoylated, they could also bind the intracellular membrane via the myristoyl chain therefore substantiating the activation of PLD through the collision mechanism.

However, the observed ARF-stimulated PLD activity in the absence of added exogenous GTPγS might also be attributed the endogenous pool of GTP that would activate recombinant ARF. This ARF activation could be mediated by residual ARF-GEFs that did not leak out during the permeabilization process. Large ARF-GEFs might be involved in guanine nucleotide exchange since their relative big size (> 100 kDa) might slow their exit from the permeabilized cells. Alternatively, an endogenous mechanism such as the NDPK might convert endogenous GDP into GTP in the presence of ATP.

More importantly still is the critical observation that myristoylation accounts for some 75% of the whole stimulatory effects of ARF on PLD activity. As a result, the remaining 25% of the total effects are attributed to the addition of GTPγS. The guanine nucleotide binding brings about a rapid and full activation of PLD by either increasing the sensitivity of ARF to PLD or activating multiple cytosolic factors. It is possible that these factors interact with PLD and affect stimulation by ARF. For instance, the stimulatory effects of the Rho family of GTPases proteins have been reported by several groups (Powner et al., 2002; Hodgkin et al., 1999; Singer et al.,
1996; Singer et al., 1995). In particular, RhoA has been shown to synergize with ARF to stimulate PLD in HL-60 cell membranes (Siddiqi et al., 1995).

Conventionally GTP binding is thought to induce the translocation of the ARF1 protein to the membrane by stabilizing a very hydrophobic conformer with an exposed, myristoylated N-terminal α-helix and therefore enhance the efficiency of PLD activation (Shome et al., 1997; Houle et al., 1995). Thus I propose that the main function of GTP in PLD activation is in translocation of ARF to membranes and the facilitation of protein-protein interactions by collision alone, rather than the induction of higher binding affinities between ARF and PLD.

The results described in this thesis also put emphasis on the presence of the amino terminus of ARFs. The N-terminus is critical for the interaction of ARF with at least one downstream effector because a deletion of this site rendered the protein unable to stimulate PLD, demonstrating that an essential interaction site on ARF, for subsequent activation of PLD, is localized to the amino terminus. This is in accord with earlier observations (Jones et al., 1999; Zhang et al., 1995). However, the failure of the recombinant deletion mutant [Δ17]ARF1 to activate PLD cannot be ascribed to failed GTPγS binding since guanine nucleotide binding assays revealed that the mutant retained this property to bind GTPγS with high affinity confirming similar observations by Jones et al. (1999) and Kahn et al. (1992). This spontaneous guanine nucleotide binding was significantly enhanced by specific GEFs such as ARNO, GRP-1 and cytohesin-1. Interestingly, the GEFs showed equal potency in catalyzing nucleotide exchange on [Δ17]ARF1. It is also true that the [Δ17]ARF1 interacts with Sec7 domain-containing ARF-GEFs present in the permeabilized cells. This result
indicates that the amino terminal α-helix of ARF1 is not essential in the interaction between the ARF and GEF proteins and therefore is not involved in the guanine nucleotide binding property of ARF (this point will be developed in the following chapter). Overall, the amino terminus of ARF is essential for ARF activity because it is an important component in coupling ARF to PLD in addition to any function mediated by switch regions in ARFs. Furthermore, the amino terminus also mediates the binding of ARF to cellular membrane phospholipids (Antonny et al., 1997) and presumably inositol lipids through the presence of hydrophobic residues and the myristate.

Finally, this chapter firmly establishes the direct comparison of the effects of ARF1 and ARF6 on PLD activity in permeabilized HL-60 cells and in vitro assays. The data clearly reveal that HL-60 PLD1 and recombinant PLD1b activities are equally regulated at low concentrations but differentially regulated at high ARF concentrations. This discrepancy was detectable at concentrations of ARF higher than 1 μM where ARF1 showed a significantly stronger PLD stimulation effect than ARF6 in both assays. Furthermore, ARF6 significantly reduced the activity of PLD at 9 μM in both model systems. Such a variance in PLD regulation by ARF1 and ARF6 is not attributed to a different degree of myristoylation because the 100% myristoylated ARF1 was still a more efficacious PLD activator than nearly 100% myristoylated ARF6 in vitro. Besides, the fact that 35% myristoylated ARF1 induced a better PLD activity than 90% myristoylated ARF6 in HL-60 cells is a reasonable proof that ARF1 is the preferred activator of PLD under the experimental conditions described in this thesis. Since this observation was made with two distinctly different assay systems it
seems very likely indeed that ARF1 is also the preferred activator in vivo. These results seem to be in agreement to some extent with some studies (Jones et al., 1999; Sung et al., 1999) but also contrast with others (Brown et al., 1995; Massenburg et al., 1994). Indeed, Sung et al. showed that 1 μM myristoylated ARF1 was a significantly better activator of PLD1 (derived from PLD1-COS-7 transfected cells) than 1 μM myristoylated ARF6 in vitro. This observation agrees with the data of this chapter on the differential activation of PLD by ARF1 and ARF6 but this discrepancy was only detected at concentrations above 1 μM ARF proteins. Using non-myristoylated ARF proteins, Jones et al. (1999) demonstrated that ARF6 was a poor activator of the baculovirus-expressed recombinant hPLD1 compared with ARF1. This difference in the stimulation of PLD activity by ARF1 and ARF6 was detectable at 10 μM ARF but was also detectable at concentrations as high as 50 μM ARF. However, given the importance of the role of N-myristoylation in PLD activation it is not clear what value can be placed on this last set of data. By contrast, Massenburg et al.’s group showed that mammalian recombinant ARF1, ARF5 and ARF6 activated one of the two PLD isoforms extractable from rat brain membranes (in a PIP2-dependent manner) with little difference in their potency. The maximal concentration levels of their ARF preparations were 3 μM for ARF1 and ARF5 and only 0.3 μM for ARF6. However, the authors did not show a direct comparison in their report. Brown et al. (1995) further suggested that the effect of a myristoylated ARF6 on preparations of PLD activity derived from HL-60 cells did not differ from that of the myristoylated ARF1 they had previously successfully characterized in an in vitro PLD assay (Brown et al., 1993). The first obvious difference between the data reported in this thesis and those
reported by Sung et al. (1999), Brown et al. (1995) and Massenburg et al. (1994) lies in the fact that the extent of myristoylation of the recombinant ARF proteins described in this thesis was accurately experimentally determined. In the previous studies the authors had not been able to discriminate the myristoylated and the non-myristoylated forms of ARFs. Therefore they could not establish what fraction of the ARF proteins used in their in vitro PLD assays were in fact myristoylated, or if they were, then to what extent. However, the degree of myristoylation of ARF1 (and probably also ARF6) as demonstrated in this thesis does not seem to affect their observed distinct effects on PLD. Nevertheless, the use of a fully myristoylated ARF protein gives a better assessment of the potential difference in the ability of the various ARF proteins to activate PLD in both permeabilized cells and in a cell-free system. Alternatively, one possible explanation for the apparent discrepancies between the data described in this chapter and those reported by other investigators might be the distinct sensitivity displayed by the PLD isoform (s) as a result of different purification procedures. Sung et al. (1999) transfected COS-7 cells with a PLD1 construct and subsequently lysed them for the assay of PLD activity in vitro. Brown et al. (1995) and Massenburg et al. (1994) extracted solubilized membrane PLD from porcine brain and rat brain respectively using differential centrifugation and detergents. In fact, Massenburg’s group separated two solubilized membrane PLD forms that were subsequently isolated by HPLC and the fractions assayed for PLD activity. One form exclusively relied on sodium oleate for activity and the other was dramatically activated by ARFs, GTP\(\gamma\)S and PIP\(_2\). Under these experimental conditions, the identity of ARF-dependent PLD was not clearly established. As a result, it is conceivable, though probably unlikely,
that the ARF-dependent PLD activity detected could have been attributed to PLD2 since PLD2 is also known to be responsive to class II ARFs (Kim et al., 2003) and also PIP2 (Colley et al., 1997). By contrast, the PLD1b isoform characterized in this thesis was expressed in sf9 cells, purified and isolated in glutathione sepharose beads. This provided large amounts of recombinant PLD1b from an expression system capable of performing many processing events such as posttranslational modifications. The enzyme was shown to be fully functional since it was readily stimulated by ARFs and PIP2 and other PLD-activating proteins such as PKCα (data not shown).

On the whole, the data reported in this chapter make a strong case for ARF1 as the preferred activator of PLD over ARF6 at high ARF concentrations. This view is readily supported by the same outcome obtained in two model systems. Importantly, it reconciles to some extent the apparent conflicting data published by Jones et al. (1999) and Sung et al. (1999) on one hand and those reported by Brown et al. (1995) and Massenburg et al. (1994) on the other. This is because at low concentrations both ARFs are activators but at high concentrations a specificity emerges.

Several reasons may explain the fact that ARF1 is the preferred activator of PLD over ARF6. Firstly, ARF1 and ARF6 have distinct subcellular distributions in many cell types. Indeed, ARF1 is thought to be predominantly cytosolic (Kahn et al., 1988) and associates with the Golgi (Palmer et al., 1993; Donaldson et al., 1992) upon GTP loading, whereas ARF6 is often localized to the plasma membrane (Caumont et al., 1998; Cavenagh et al., 1996; Peters et al., 1995) and endosomes (Peters et al., 1995). Secondly, human ARF1 and human ARF6 are different in sequence, being only 66% identical at the amino acid level (Tsuchiya et al., 1991). The crystal structures of
ARF1 and ARF6 have been determined in several forms (Menetrey et al., 2001; Menetrey et al., 2000; Goldberg, 1998; Greaslet et al., 1995; Amor et al., 1994). Menetrey et al. (2000) showed that ARF1 and ARF6 have different conformations in their GDP-bound form. This finding indicates that these two proteins might have distinct guanine nucleotide binding properties. Interestingly, the data described in the next chapter showed that the rate of binding of guanine nucleotide catalysed by the small GEFs was higher on ARF1 than on ARF6 therefore suggesting a more specific molecular interaction between the ARF1 and the GEFs. As a result, this observation reinforces the view that PLD is preferentially regulated by ARF1 and this regulation is a function of its guanine nucleotide binding activity mediated by the small GEFs. This point will be considered in more details in the next chapter.

Taken together, the data described in this chapter clearly highlight myristoylation as a critical feature in the activation of PLD, one of the several ARF functions. Therefore optimal data in biochemical assays using recombinant ARF proteins are highly dependent on the generation of fully-myristoylated ARF proteins and failure to achieve this may devalue the relevance of any data obtained. The N-terminus of ARF is an essential interacting site for PLD. Finally, the comparison of the effects of ARF1 and ARF6 on PLD activity strongly points to a role for ARF1 and not ARF6 as the factor that regulates PLD1 in permeabilized cell and cell-free model systems and therefore probably also in vivo. However, this apparent discrepancy between ARF1 and ARF6 in the regulation PLD activity in intact cells has not been established yet.
Specificity of small ARF-GEFs for both ARFs and polyphosphoinositides
4.1. Introduction

Stimulation by agonists, hormones, growth factors and neurotransmitters promotes the activation of one or more regulators of PLD in most cell types (Carpio and Dziak, 1998; Shome et al., 1997; Garcia et al., 1992; Billah et al., 1989; Liscovitch et al., 1989). In the case of the ARF family proteins, activation results from the exchange of GDP for GTP on ARFs. This exchange reaction can occur spontaneously but it is greatly accelerated specific GEFs. The small GEFs involved in the regulation of the ARF proteins include all ARNO/GRP-1/cytohesin-1 members (Donaldson and Jackson, 2000; Jackson and Casanova, 2000). These GEFs possess a specific phosphoinositide-binding site commonly known as the pleckstrin homology (PH) domain. Therefore, these low molecular weight GEFs were shown to be specifically regulated by polyphosphoinositides (Klarlund et al., 2000; Klarlund et al., 1998; Klarlund et al., 1997; Chardin et al., 1996), some of which are involved in the regulation of PLD (Hammond et al., 1997; Brown et al., 1993).

This chapter will focus on the role of GEFs in activating ARF proteins and their specific interactions with polyphosphoinositides. These polyphosphoinositides were previously shown to be implicated in an array of cellular functions by coordinating the transient recruitment of their target proteins to the site of activation of downstream specific effector proteins.
4.2. Results

4.2.1. Purification of small ARF-GEFs proteins

Procedure for the purification of the small ARF-GEFs, ARNO, GRP-1 and cytohesin-1 is described in the Materials and Methods section. His-tagged ARNO, GRP-1 and cytohesin-1 proteins obtained after cell lysis were subjected to chromatography on Ni-NTA column and subsequently eluted with a linear gradient of 0 to 0.5 M imidazole. Samples of proteins in the collected active fractions were separated by SDS-PAGE and subsequently stained with Coomassie blue (Figure 4.1). Bands were detected around 47 kDa.

4.2.2. Novel guanine nucleotide binding assay

During the course of my investigation into the regulation of ARF activity by the small ARF-GEFs, I have developed a novel guanine nucleotide binding assay. This assay readily demonstrates the stimulation of [35S]GTPγS binding to ARF proteins, the exchange reaction can occur either spontaneously or greatly accelerated in the presence of the GEFs (see below). Because the nucleotide exchange assay is based on the binding of His-tagged proteins to Ni-NTA agarose beads, it is necessary to estimate the quantity of ARF proteins that is recovered on beads following a period of exposure to the beads. Figure 4.2. shows that an average of 20 to 25% of ARF proteins were recovered on beads under the experimental conditions described in this thesis. This estimate allows calculation of the fraction of ARF protein present that undergoes exchange.
Figure 4.1. Purification of small ARF-GEF proteins. The supernatant obtained after cell lysis was applied to a 25 mL bed volume column of Ni-NTA, which had previously been equilibrated in 100 mL of Nickel column buffer (20 mM NaH₂PO₄, 20 mM Tris, 300 mM NaCl, 0.2% NaN₃ (w/v), pH 6.0) followed by 100 mL of the same buffer supplemented with 0.5 M imidazole and then followed by 50 mL of a mixture (5:2 ratio) of lysis buffers 1 and 2 respectively (see Materials and Methods section for more details). Proteins were eluted with a linear gradient of 0 to 0.5 M imidazole in the Nickel column buffer. Fractions of 5 mL were collected. Protein elution was monitored by absorbance at 280 nm. Samples of the corresponding eluted fractions were analyzed by SDS-PAGE and Coomassie blue staining.
Figure 4.2. Recovery of myristoylated ARF1 on Ni-NTA beads. Different concentrations of myristoylated ARF1 were exposed to 25% slurry beads for 30 min. ARF1 proteins were eluted off the beads with imidazole and analyzed by SDS-PAGE (upper panel) and Western blot (lower panel) as described in Materials and Methods section. 1 μM ARF in a GTPγS binding experiment represents approximately 21 μg/mL.
4.2.3. Myristoylation is necessary but not essential in enhancing the guanine nucleotide exchange on ARF proteins

ARNO, GRP-1 and cytohesin-1 were first examined for their guanine nucleotide exchange activity on [Δ17]ARF1 (ARF1 with the N-terminal 17 amino acid residues deleted), 35% myristoylated ARF1 and 90% myristoylated ARF6. The in vitro assay to monitor the exchange activity of the GEFs on ARFs is based on the binding of radio-labeled guanine nucleotides in the presence of artificial lipid vesicles supplemented with PIP2 in near physiological conditions of salt and pH. The lipid vesicles were included in these assays because previously ARF and GEF proteins have been shown to interact with membrane lipids through their myristoylated N-terminus and the PH domain respectively (Beraud-Dufour et al., 1999; Antonny et al., 1997). PIP2 was also added to the phospholipid vesicles as this phosphoinositide was reported to enhance the nucleotide exchange activity of the GEFs (Paris et al., 1997; Chardin et al., 1996). The three recombinant ARFs were tested simultaneously as shown in Figure 4.3. For both ARF1 and ARF6 there was a spontaneous GTPγS binding but the extent was much greater for 90% myristoylated ARF6 than for 35% myristoylated ARF1 (P = 0.02, n = 3). However, the rate of spontaneous binding to ARF1 was apparently increased by the deletion of the N-terminal α-helix ([Δ17]ARF1) although this increase was not significant (P = 0.07, n = 3). The rate of guanine nucleotide exchange on [Δ17]ARF1, 35% myristoylated ARF1 and 90% myristoylated ARF6 was then significantly augmented in the presence of all three GEFs tested (P < 0.05, n = 3). Indeed, the GEFs effectively enhanced the spontaneous loading of GTPγS 3 to 4 times on [Δ17]ARF1, 8 times on 35% myristoylated ARF1 and 3 times on 90%
Figure 4.3. GEF specificity binding of ARNO, GRP-1 and cytohesin-1 for ARF proteins. Specific binding of [35S]GTPγS to [Δ17]ARF1 (1 μM), 35% myristoylated mARF1 (1 μM) and 90% myristoylated mARF6 (1 μM) was determined after 1 min incubation at 37°C in exchange buffer containing 50 mM Hepes, pH 7.5, 1 mM MgCl₂, 100 mM KCl, 1 mM DTT, 12 μM [35S]GTPγS and 1 mM phospholipid vesicles in 50 μl volume of reaction where the present GEFs were at a final concentration of 1 μM. Data are means ± S.E.M., n= 3 experiments. Asterisks indicate significantly different from the control (P < 0.05).
myristoylated ARF6. These results suggest that the GEFs seem to be relatively more active on ARF1 than on ARF6 and [Δ17]ARF1.

The effect of increasing the extent of myristoylation was investigated next. The guanine nucleotide exchange reaction catalysed by all three tested members of the family of ARNO-like proteins was effective on myristoylated and non-myristoylated forms of ARF1 and ARF6 in the presence of phospholipid vesicles supplemented with 2% PIP2. As illustrated in Figure 4.4(A), the binding of [35S]GTPγS to 35% myristoylated ARF1 and its non-myristoylated counterpart was equally and significantly increased by the GEFs (P < 0.05, n = 3) except for cytohesin-1 whose apparent exchange activity on the non-myristoylated ARF1 was not significantly different from the spontaneous binding of [35S]GTPγS to non-myristoylated ARF1 (P = 0.17, n = 3). The guanine nucleotide exchange catalyzed by ARNO and GRP-1 on 35% myristoylated ARF1 was significantly greater than on the non-myristoylated ARF1 (P = 0.02, n = 3). However, the guanine nucleotide exchange mediated by cytohesin-1 was not significantly different on these two forms of ARF (P = 0.27, n = 3). In addition, the comparison of the 100% and 35% myristoylated ARF1 proteins further demonstrated that increasing the percentage of myristoylation on ARF protein also significantly enhanced the nucleotide exchange rate on ARF (P < 0.05, n = 3). Indeed, Figure 4.4(B) showed that the exchange reaction on the 100% myristoylated ARF1 was 4 to 6 times greater than on the 35% myristoylated ARF1 (P < 0.05, n = 3). Likewise, the exchange reaction on the 90% myristoylated ARF6 was about two-fold more effective than on the non-myristoylated ARF6 (Figure 4.4(C)). In addition, ARF1 and ARF6 were comparable in their ability to support guanine nucleotide exchange
Figure 4.4. GTPγS binding to recombinant ARF proteins catalyzed by GEFs is increased by myristoylation. Specific binding of [35S]GTPγS to ARF proteins (1 μM) was determined after 5-10 min incubation at 37°C in exchange buffer containing 50 mM Hepes, pH 7.5, 1 mM MgCl2, 100 mM KCl, 1 mM DTT, 12 μM [35S]GTPγS and 1 mM phospholipid vesicles supplemented with 2% PIP2 in 50 μl volume of reaction where the present GEFs were at a final concentration of 1 μM. Data are means ± S.E.M., n= 3 experiments. Data are means ± S.E.M., n= 3 experiments. Asterisks indicate significantly different from the control (P < 0.05).
exchange activity. Although 90% myristoylated ARF6 was used at the time of the experiments, it is safe to assume that a further 10% increase in myristoylation would render 100% myristoylated ARF6 as efficient as 100% myristoylated ARF1 in its ability to bind $[^{35}\text{S}]$GTP$\gamma$S.

All the tested small ARF-GEFs were efficient in catalysing the guanine nucleotide exchange reaction on the studied ARF proteins although cytohesin-1 seemed to catalyse a weaker nucleotide exchange on the 35% myristoylated ARF1. However, this observation was not always consistent as preliminary data showed that other preparations of cytohesin-1 were as efficient as ARNO and GRP-1 in their capacity to stimulate the nucleotide exchange on ARF1 and ARF6.

4.2.4. The isolated PH domains and full length ARF-GEFs interact specifically with polyphosphoinositides

The binding of the studied small ARF-GEFs to polyphosphoinositides were independently and quantitatively demonstrated by using a liposome-binding assay. In this assay, the recombinant ARNO, GRP-1 and cytohesin-1 proteins were separately mixed with large unilamellar liposomes produced by the extrusion technique. The liposomes contain the indicated phosphoinositides and the GEFs were shown to bind following ultracentrifugation of the protein-lipid mixtures. As shown in Figure 4.5, each of the PH domains bound specifically to PI(3,4)P$_2$, PI(4,5)P$_2$, and PIP$_3$. The PH domains of ARNO and GRP-1 showed between 25% and 45% binding to these particular phosphoinositides, whereas about 45% of the PH domain of cytohesin-1 bound to the mentioned phosphoinositide-containing vesicles at a total lipid concentration of 2 mM, corresponding to 60 $\mu$M phosphoinositides. The extent of
Figure 4.5. Phosphoinositide binding specificity of the GEFs PH domains. Binding of the isolated PH domains of ARNO (panel A), GRP-1 (panel B) and cytohesin-1 (panel C) to liposomes made of neutral phospholipids PC and PS (4:1 molar ratio) supplemented with 3% of total lipids of various phosphoinositides as indicated. The exposure of the PH domains of the GEFs to the liposomes were performed at room temperature for 1 hour. The liposomes were pelleted by ultracentrifugation at 25,000 x g for 1 hour at 25°C. The amount of protein that remained in the supernatant was quantified using a dye-binding assay based on the method of Bradford and compared with that of a control reaction that contained proteins without lipids. Error bars represent the standard error of the mean (SEM) for three independent experiments (n = 3). The significance of data was evaluated using Student’s t test.
binding of the PH domains of ARNO and cytohesin-1 to PI(3,4)P₂, PI(4,5)P₂, and PIP₃ was not significantly different from each other (P > 0.05, n = 3). The PH domain of GRP-1 preferentially bound PI(3,4)P₂ over PI(4,5)P₂ (P = 0.04, n = 3) but its binding affinity towards PIP₃ was not significantly different from that shown towards PI(3,4)P₂ (P = 0.10, n = 3) (Figure 4.5 (B)). Very weak binding was observed with the other phosphoinositides namely PI(3)P, PI(4)P and PI(5)P. Interestingly, the isolated PH domains also bound PI(3,5)P₂. The binding affinity towards this phosphoinositide was about two to three times smaller than that observed for PI(3,4)P₂, PI(4,5)P₂, and PIP₃. The PH domains of GRP-1 and cytohesin-1 especially showed a significantly stronger binding towards PI(3,5)P₂ than that of ARNO (P < 0.05, n = 3) (Figure 4.5 (A), (B) and (C)).

Likewise, the full-length small ARF-GEFs also displayed a strong binding affinity for PI(3,4)P₂, PI(4,5)P₂, and PIP₃. As illustrated in Figure 4.6, the full-length ARNO and cytohesin-1 showed similar binding potency towards PI(3,4)P₂, PI(4,5)P₂, and PIP₃ whereas the full-length GRP-1 bound significantly better to PI(3,4)P₂ than to PI(4,5)P₂ (P = 0.01, n = 3). Very little or no binding was observed when the full-length GEFs were incubated with PI(3)P, PI(4)P and PI(5)P although ARNO showed a significantly higher percentage binding to these phosphoinositides than GRP-1 and cytohesin-1 (P > 0.05, n = 3). Most interestingly, the observed binding of the GRP-1 and cytohesin-1 PH domains to PI(3,5)P₂ was completely abolished by using full-length GEF proteins. The percentage of proteins bound to PI(3,5)P₂ decreased 20-fold and 5-fold when the full-length GRP-1 and cytohesin-1 were respectively exposed
Figure 4.6. Phosphoinositide binding specificity of the full-length GEFs. Binding of the full length ARNO (panel A), GRP-1 (panel B), and cytohesin-1 (panel C) to liposomes made of neutral phospholipids PC and PS (4:1 molar ratio) supplemented with 3% of total lipids of various phosphoinositides as indicated. The exposure of the GEFs to the liposomes were performed at room temperature for 1 hour. The liposomes were pelleted by ultracentrifugation at 25,000 x g for 1 hour at 25°C. The amount of protein that remained in the supernatant was quantified using a dye-binding assay based on the method of Bradford and compared with that of a control reaction that contained proteins without lipids. Error bars represent the standard error of the mean (SEM) for three independent experiments (n = 4). The significance of data was evaluated using Student's t test.
to liposomes containing PI(3,5)P\(_2\). By contrast, the full-length ARNO showed the same binding affinity for PI(3,5)P\(_2\) as its isolated PH domain.

The high affinity specific binding of the PH domains and full-length ARNO, GRP-1 and cytohesin-1 for PI(3,4)P\(_2\), PI(4,5)P\(_2\), and PIP\(_3\) observed in the liposome binding assay were further qualitatively confirmed by similar results obtained using the protein-lipid overlay or dot-blot assay. As shown in Figures 4.7, 4.8 and 4.9, the PH domains and full-length GEFs displayed a clear specificity for PI(3,4)P\(_2\), PI(4,5)P\(_2\) and PIP\(_3\) as shown by high-intensity binding signals. However, the binding of the full-length ARNO and the cytohesin-1 PH domain to PIP\(_3\) was not clearly detected (Figures 4.7 and 4.9). This observation along with the fact that neither the isolated PH domains nor the full-length GEFs showed binding specificity towards PI(3,5)P\(_2\) in this type of assay indicate a possible removal of the phosphoinositides applied to the Hybond-C extra-membranes during the blocking and the washing steps. Alternatively, the binding interactions on vesicle surfaces may be different from those with polyphosphoinositides dispersed on nitrocellulose.
Figure 4.7. Phosphoinositides-binding properties of the full-length ARNO and its isolated PH domain. The ability of the full-length 6xHis-tagged ARNO and its GST-Cys-tagged PH domain to bind a variety of phosphoinositides was analysed using a protein-lipid-binding assay. Serial dilutions of indicated phosphoinositides (200, 100, 50, 25, 12.5 and 6.25 pmol) were spotted on to Hybond-C extra membranes, which were then incubated with the purified recombinant 6xHis-tagged or Cys-tagged proteins. The membranes were washed, and the 6xHis-tagged and Cys-tagged proteins bound to the membranes were detected using and anti-His or anti-GST antibody.
Figure 4.8. Phosphoinositides-binding properties of the full-length GRP-1 and its isolated PH domain. The ability of the full-length 6xHis-tagged GRP-1 and its GST-Cys-tagged PH domain to bind a variety of phosphoinositides was analysed using a protein-lipid-binding assay. Serial dilutions of indicated phosphoinositides (200, 100, 50, 25, 12.5 and 6.25 pmol) were spotted on to Hybond-C extra-membranes, which were then incubated with the purified recombinant 6xHis-tagged or Cys-tagged proteins. The membranes were washed, and the 6xHis-tagged and Cys-tagged proteins bound to the membranes were detected using and anti-His or anti-GST antibody.
Figure 4.9. Phosphoinositides-binding properties of the full-length cytohesin-1 and its isolated PH domain. The ability of the full-length 6xHis-tagged cytohesin-1 and its cytohesin-1-Cys-tagged PH domain to bind a variety of phosphoinositides was analysed using a protein-lipid-binding assay. Serial dilutions of indicated phosphoinositides (200, 100, 50, 25, 12.5 and 6.25 pmol) were spotted on to Hybond-C extra-membranes, which were then incubated with the purified recombinant 6xHis-tagged or Cys-tagged proteins. The membranes were washed, and the 6xHis-tagged and Cys-tagged proteins bound to the membranes were detected using an anti-His or anti-GST antibody.
4.3. Discussion

This chapter concentrates on the molecular basis of the regulation of ARF proteins by the family of small ARF-GEFs and polyphosphoinositides. All three members of the small GEFs family i.e. ARNO, GRP-1 and cytohesin-1 described in this thesis stimulated with the same degree of efficiency the guanine nucleotide exchange on the different forms of ARF1 and ARF6 proteins.

The data showing that the deletion of N terminal of ARF1 where myristate is post-transtonally added did not affect the rate of GTP exchange either arising spontaneously or accelerated by the GEFs confirms previous observations (Pacheco-Rodriguez et al., 1998; Paris et al., 1997). These results indicate that the N-terminus-truncated mutant of ARF1 can still interact with the GEFs despite the fact that [Δ17]ARF1 cannot bind to phospholipid vesicles on which the physical interaction between the ARF and GEF proteins are thought to occur (Jackson and Casanova, 2000). This interaction with the GEFs does not involve the N-terminal of the ARF protein. This observation was confirmed by structural studies in which a model of the interaction between ARF1-GDP and the Sec7 domain of ARNO was proposed (Beraud-Dufour et al., 1998). According to this model, the N-terminal helix of ARF1 did not physically interact with the ARNO-Sec7. The N-terminal helix of ARF1 had in fact been shown to be buried in a pocket at the surface of ARF1. Based on their own studies and those of others (Betz et al., 1998; Cherfils et al., 1998; Goldberg, 1998; Mossessova et al., 1998), Beraud-Dufour et al. (1998) proposed a model for the mechanism of the guanine nucleotide exchange catalyzed by ARNO-Sec-7 on [Δ17]ARF1. The GDP/GTP exchange is dependent on the interaction of the switch
regions of ARF and the catalytic Sec7 domain of the GEFs. A conserved glutamate residue (E156) in the ARNO-Sec7 domain, located in a hydrophobic groove is strongly involved in the exchange mechanism because a mutation of glutamate to lysine (E → K) or to aspartate (E → D) resulted in a dramatic decrease in the exchange activity of ARNO-Sec7 on ARF1 (Cherfils et al., 1998). This glutamate residue contributes to the release of the GDP by interacting with the Mg bound to ARF through the negatively charged carboxylate group of the glutamate side-chain. This interaction would result in the destabilization of the Mg coordination with the β-phosphate of GDP leading to the removal of the Mg$^{2+}$. The spontaneous dissociation of the nucleotide would then be brought about by electrostatic repulsion. Thus this model could be generalized to the other two ARF-GEFs, namely GRP-1 and cytohesin-1 since the glutamate and surrounding residues are conserved in all three proteins.

*In vitro* experiments comparing non-myristoylated, partially and fully myristoylated forms of ARF proteins clearly demonstrated that myristoylation significantly increases nucleotide exchange efficiency on ARF proteins catalysed by the GEFs. However, this is achieved only when the ARF-GEF interactions occur in the presence of phospholipid vesicles supplemented with PIP$_2$. This is in agreement with previous reports (Paris et al., 1997; Chardin et al., 1996). In the normal, physiological, cellular context, the fully-myristoylated form of ARF is the only form of the proteins found. Upon activation of ARF by the GEFs, it is generally thought that the previously buried N-terminal α-helix of ARF becomes exposed to the solvent. The subsequent binding of ARF to the cellular membrane promoted by this increased surface
hydrophobicity, due to the presence of the myristoyl group, confers a high affinity for the lipid membrane. This initial binding would be further strengthened by the interaction of the hydrophobic residues of the N terminal helix of ARF-GTP with the lipid bilayer (Antonny et al., 1997).

The data presented in this thesis showed that ARNO, GRP-1 and cytohesin-1 were all equally efficient in stimulating the nucleotide exchange on both ARF1 and ARF6 in vitro. ARNO was originally recognized as a GEF for ARF1 in vitro and was thought to be the principal GEF for ARF1 (Chardin et al., 1996). Subsequent in vitro nucleotide binding studies based on radio-labeled nucleotides and highly myristoylated ARF forms (= 80% myristoylated) also favoured ARNO as a GEF for ARF1 over ARF6 (Franco et al., 1998). These results support those reported by Macia et al. (2001) and Beraud-Dufour and Robineau (2001) who used a real-time assay to monitor the exchange activity on the myristoylated ARF1 and ARF6 proteins. This assay is based on the difference between the tryptophan fluorescence of ARF-GDP and of ARF-GTP. Using this assay Macia et al. (2001) demonstrated that the nucleotide exchange catalysed by ARNO was 5 times greater on myristoylated ARF1 than myristoylated ARF6. By contrast, Frank et al. (1998) showed that ARNO was a better GEF for ARF6 than for ARF1 using a classical [35S]GTPγS exchange assay. This apparent discrepancy is most likely due to the fact that the ARF forms used in the in vitro assays differ in the degree of myristoylation. Frank et al. (1998) used 20% myristoylated ARF1 and 50% myristoylated ARF6 whereas Franco et al. (1998) employed at least 80% myristoylated ARF1 and ARF6. The extent of myristoylation of the ARFs was not determined in the studies reported by Macia et al. (2001). Frank
et al. (1998) further demonstrated that ARNO and ARF6 co-localized to the plasma membrane of transfected cells. This observation was interpreted as evidence supporting a role for ARNO as a GEF for ARF6 rather than ARF1 in cells. Several other groups showed that ARNO can be expressed either at the Golgi (Lee et al., 2000; Monier et al., 1998) in which case ARNO would preferentially activate ARF1, or at the plasma membrane (Caumont et al., 2000; Venkateswarlu et al., 1998) or endosomal membranes (Maranda et al., 2001) where ARNO would probably associate with ARF6 since this isoform is thought to be enriched in this region (Cavenagh et al., 1996, Peters et al., 1995). The detection of in vivo nucleotide exchange on ARF6 catalysed by ARNO (Santy et al., 2001) further corroborates the argument that ARNO is a suitable candidate in ARF6 activation in cells. Overall, the localisation of ARNO and its substrate specificities depend on experimental conditions and the type of cells under investigation. There seems to be a consensus that in the absence of the GEFs the in vitro spontaneous nucleotide exchange on ARF6 is faster than on ARF1 (Macia et al., 2001). This finding might indicate that a larger fraction of ARF6 is spontaneously bound to membranes in vivo compared to ARF1.

In vitro studies demonstrated that GRP-1 is the principal GEF for ARF1 (Franco et al., 1998; Klarlund et al., 1998). The authors of these studies showed that GRP-1 could also function as a GEF for ARF5 to a lesser extent but failed to activate ARF6. Furthermore, Franco et al. (1998) overexpressed GRP-1 in mammalian cells. As a result, a disruption of the Golgi function was observed. This finding prompted these authors to propose that ARF1 is the major substrate for GRP-1 in vivo. However, in contrast to this view, Langille et al. (1999) showed that GRP-1 could activate ARF6
using a similar in vitro nucleotide binding assay to that employed by Franco et al. (1998). In addition to the cell-free system, Langille et al. (1999) also used intact cells to demonstrate that GRP-1 could stimulate the nucleotide loading on both ARF1 and ARF6 in these cells. Because the co-localization of GRP-1 with ARF6 at the plasma membrane of transfected cells was observed, the authors of this report suggested a physiological role for GRP-1 in regulating ARF6 functions in cells.

Among the ARFs so far tested in vitro, cytohesin-1 was shown to activate ARF1 (Ogasawara et al., 2000; Franco et al., 1998; Pacheco-Rodriguez et al., 1998; Meacci et al., 1997), ARF3 (Meacci et al., 1997), but had no effect on ARF6 (Pacheco-Rodriguez et al., 1998). The results described in this thesis clearly contradict these previous observations and show that cytohesin-1 can function as a GEF for ARF6 in vitro. This particular difference might be ascribed to the low degree of myristoylation of the ARF6 protein used in Pacheco-Rodriguez's studies. However, cytohesin-1 has been shown to translocate to the plasma membrane following stimulation of PI 3-kinase in transfected cells (Venkateswarlu et al., 1999). Therefore, cytohesin-1 might very well interact with ARF6 in vivo using the same criteria of inference as Langille et al. (1999).

Overall, the data presented in this thesis combined with past and recent reports reveal that ARNO, GRP-1 and cytohesin-1 can display activity for ARFs from all three classes in both in vitro and in vivo experiments. However, differences in in vitro assay conditions, such as Mg\(^{2+}\) concentration, the type of assays used to monitor the nucleotide exchange, the methods used to produce lipid vesicles with or without the incorporated phosphoinositides, the degree of myristoylation of the purified ARFs, the
types of cells used for in vivo experiments can account for the difficulty in identifying a precise physiological substrate for each of the characterized GEFs.

The ARNO/GRP-1/cytohesin-1 family possess a C-terminal PH domain which is a common feature of the GEFs. Therefore these GEFs are potential candidates for specific interaction with phosphoinositides which were widely shown to be implicated in the regulation of PH-domain containing proteins (Jackson et al., 2000; Venkateswarlu et al., 1999; Klarlund et al., 1998; Venkateswarlu et al., 1998). The results presented in this thesis quantitatively and qualitatively demonstrate that both the full-length and the isolated PH domains of the GEFs bind with equally high affinity and specificity to PI(3,4)P₂, PI(4,5)P₂, and PIP₃. However, these data seem to contradict previous reports showing for instance that the GRP-1 PH domain binds PIP₃ with high degrees of affinity and selectivity over PI(3,4)P₂ and PI(4,5)P₂ in in vitro binding assays (Klarlund et al., 2001; Klarlund et al., 2000; Kavran et al., 1998; Klarlund et al., 1998; Klarlund et al., 1997) and structural studies (Ferguson et al., 2000; Lietzke et al., 2000). Indeed, the soluble PIP₃ head group, Ins(1,3,4,5)P₄ (Kavran et al., 1998) or soluble C₈ PIP₃ (Klarlund et al., 2001; Klarlund et al., 2000; Klarlund et al., 1998) were shown to bind with higher specificity to GRP-1 PH domain than to ARNO and cytohesin-1 PH domains in phosphoinositide binding and competition assays. Furthermore, Klarlund et al. (1998) had previously tested PI(3,4)P₂, PI(4,5)P₂, and PIP₃ for their effects in stimulating GRP-1-mediated exchange activity of ARF1. The selectivity of PIP₃ in enhancing ARF1 nucleotide exchange by GRP-1 over that observed for PI(3,4)P₂ or PI(4,5)P₂ had been detected at lower charge density in their phospholipid micelle-based assay. In addition, the same
authors had shown that the PIP3-mediated ARF1 nucleotide exchange through GRP-1 was specifically inhibited by Ins(1,3,4,5)P4 while the other polar head groups tested, including Ins(1,3,4)P3, Ins(1,4,5)P3, Ins(1,3,4,6)P4 and Ins(1,2,5,6)P4 did not block the stimulation of ARF1 exchange activity by GRP-1. A closer examination of the amino acid sequence comparison of the PH domain of ARNO, GRP-1 and cytohesin-1 show that the three PH domains used in the experiments described in this thesis and those reported by Klarlund et al. (2000) are almost identical (Figure 4.10). Indeed, the sequence alignment reveals that the GRP-1 PH domain possesses a diglycine motif which differs from ARNO and cytohesin-1 PH domains which harbour a triglycine motif. As a consequence, Klarlund et al. (2000) suggested that the unusual diglycine motif in the GRP-1 PH domain, as opposed to the triglycine in ARNO and cytohesin-1 provides a possible explanation for the highly PIP3 binding selectivity displayed by GRP-1 PH domain. A point mutation in which an additional glycine was added to the GG motif of the GRP-1 PH domain greatly increased its binding affinity for PI(4,5)P2 compared with the native protein while it decreased its binding affinity for PIP3. Conversely, a mutant ARNO PH domain in which a single glycine was deleted in the GGG motif led to a reduced binding affinity for PIP2 but not for PIP3. Therefore Klarlund et al. (2000) concluded that the binding selectivity of GRP-1 PH domain towards PIP3 may be attributed to the unique presence of the diglycine motif as opposed to the triglycine in ARNO and cytohesin-1 PH domain.

The results presented in this thesis and those reported by other investigators are indeed contradictory. Different experimental conditions and different models used to investigate the binding properties of the studied GEFs might provide an explanation
Figure 4.10. Comparison of the sequences corresponding to the PH domains of cytohesin-1, ARNO and GRP-1. Comparison of the sequences of the PH domains correspond to residues 208 to 324 of the full-length ARF-GEFs. Small portions of sequence corresponding to GST, from the PH-domain C-terminal fusion protein constructs prepared for experiments described in this thesis, are also included in the alignment, exactly as they were detected during sequencing. Diglycine (GG) and triglycine (GGG) motifs are visible between positions 218 and 220 as shown by asterisks. Alignment was generated by ClustalW and the printing of the multiple alignment output was performed by Boxshade.
for this apparent discrepancy. The lipid binding assay described by Klarlund et al. (2000) was based on the use of GST/PH domain fusion proteins bound to glutathione immobilized on agarose beads to which were added labeled or unlabeled phosphoinositides with eight carbon atoms in the fatty acyl chains. In contrast, the lipid-binding assay described in this thesis is based on the production of large unilamellar liposomes as the “support” for the various phosphoinositides. These phosphoinositides carry fourteen carbon atoms in their hydrophobic chains. As a result the lipids used in my investigations were dispersed in a model membrane rather than as non-physiological monomers in bulk aqueous solvent. Notably, other investigators have examined di-glycine PH domain binding to large vesicles and found that any observed specificity for PIP3 over PIP2 was at most 3 to 4 fold enhanced rather than the 650-fold shown by Klarlund et al. (2000). Additionally, these investigations indicated the sensitivity of binding to pH and salt concentration.

Whether the demonstrated differential polyphosphoinositide specificity is delivered exclusively by the PH domain, as Klarlund et al. (2000) suggest, remains to be firmly established. However, the data reported in this thesis clearly show that the full-length GEFs usually displayed the same binding characteristics as their respective isolated PH domains which exhibit very high sequence similarity. Therefore the distinct polyphosphoinositide binding selectivity reported for GRP-1 might not be located wholly in the PH domain. Despite these differences observed for the binding properties of the GEFs for the major phosphoinositides, the general consensus is that the studied GEF PH domains can bind with high affinity to P(3,4)P2, PI(4,5)P2, PIP3, and to a lesser extent PI(3,5)P2. Because some of these phosphoinositides are
generated through the action of a family of PI 3-kinases (Rameh and Cantley, 1999; Toker and Cantley, 1997), it is therefore plausible to suggest a role for PI 3-kinases enzymes in the regulation of the mechanisms of recruitment and subsequent activation of ARF proteins leading ultimately to the stimulation of PLD activity (Rizzo and Romero, 2002). The activation of isoforms of type I PI 3-kinases such as p85α/β- p110α/β/δ and p110γ type PI 3 kinases leads to rapid phosphorylation of the inositol D-3 positions on PI, PI(4)P, and PI(4,5)P2 (Zvelebil et al., 1996). Receptor stimulation by insulin or growth factors that link to the PI 3-kinase pathway was shown to induce the recruitment of the GRP-1 (Klarlund et al., 2000), ARNO (Venkateswarlu et al., 1998) and cytohesin-1 (Venkateswarlu et al., 1999) to the plasma membrane. The observed PH domain-dependent translocation of these GEFs to the plasma membrane was inhibited by chemically unrelated PI 3-kinase inhibitors wortmannin (Okada et al., 1994) and LY294002 (Vlahos et al., 1994).

Taken together, the data presented in this thesis and those mentioned above still support a participation of the PI 3-kinase pathway in the activation of PLD following the sequential recruitment and physical interaction of the GEFs and ARFs on intracellular membranes. However, the ability of newly generated 3-phosphorylated lipids to recruit these GEFs in the standing presence of vastly greater amounts of the equally well-bound PI(4,5)P2 (from now on referred as PIP2) remains to be explained.

Interestingly, recent findings have complicated the common model mentioned above that GEFs translocate as a result of binding to phosphoinositides generated by agonists binding to plasma membrane. Indeed, the GEFs have been shown to interact with partner proteins that modulate its localization and potentially their functions. For
instance, it has been demonstrated that GRP-1 could bind in particular to two novel
proteins termed GRSP1 (GRP-1 signaling partner 1) (Klarlund et al., 2001a) and
GRASP also known as tamalin (Kitano et al., 2002; Nevrivy et al., 2000) in vitro. The
interaction of GRP-1 with these two proteins is mediated by its the N-terminal coiled-
coil domain. In addition, GRP-1 was shown to interact with GRSP1 and GRASP in
co-transfected mammalian COS-1 and HEK293 cells, respectively. In particular, these
two interacting proteins co-localize with GRP-1 at the plasma membrane of
transfected cells. Interestingly, co-localization of ARFs and GRASP was also
observed at the plasma membrane of transfected cells. This particular observation
indicates a possible role of GRASP in ARF signaling. In addition to GRP-1, ARNO
and cytohesin-1 can also interact and co-localize with binding partners. Indeed, like
GRP-1, ARNO strongly interacts with GRASP in vitro while cytohesin-1 has been
shown to bind Munc13-1, a presynaptic protein that is translocated to diacylglycerol-
containing membranes in presynaptic active zones (Neeb et al., 1999). In the light of
these new findings, Klarlund et al. (2001) and Nevrivy et al. (2000) suggested that
GRSP1 and GRASP function as adaptor proteins that selectively target the GEFs to
discrete plasma membrane domains in response to stimulation by agonists. However,
it is still unclear whether these novel interacting proteins further enhance the specific
recruitment of the GEFs to membranes mediated by phosphoinositides or act
independently in response to the stimulation of other signaling pathways.
Efficient coupling of GEFs-ARFs-PLD1 in permeabilized HL-60 cells is not preserved in vitro: role of guanine nucleotides (GDP, GTP) and polyphosphoinositides (PIP$_2$, PIP$_3$)
5.1. Introduction

As discussed in Chapter 3, ARF1 turns out to be the preferred activator of PLD over ARF6 both in permeabilized HL-60 cells and in a cell-free system. *In vitro* assays demonstrated that the efficient activation of ARFs was enhanced by the presence of the small ARF-GEFs as demonstrated in Chapter 4. These exchange factors effectively catalyzed the exchange of GDP for GTP on ARFs with similar potency. In the two preceding chapters the interaction of these two complexes (GEF + ARF, ARF + PLD) were studied independently, an approach similar to that taken by many other investigators. However, physiologically these two "half-reactions" may in reality form part of a common pathway leading to the effective stimulation of PLD activity in cells. Polyphosphoinositides are critical signalling molecules in the PLD pathway in that besides supporting basal PLD activity one of their proposed functions is to recruit the PH domain-containing GEFs to membranes where they would in turn recruit ARFs.

The purpose of the experiments described in this chapter is to attempt to conciliate these two "half-reactions" into one ternary complex both in permeabilized HL-60 cells and in cell-free assays and verify whether the tested GEFs display any specificity in activating PLD through ARF1 in these two systems. The role of PIP$_2$ and PIP$_3$ was also assessed for their effects on the coupling between GEFs, ARFs and PLD.

To the best of my knowledge, the properties of the combined ternary complex comprising GEF, ARF and PLD has never been examined in either permeabilized HL-60 cells or cell-free systems.
5.2. Results

5.2.1. Stimulation of PLD activity by ARF1 is increased in the presence of the GEFs in permeabilized HL-60 cells but the same GEFs do not augment ARF-stimulated PLD activity in cell-free systems

The relative PLD activation efficiency of the GEFs was compared using two related experimental conditions. Concentrations of the GEFs were normalized to each other using firstly the same absolute protein concentration (Figure 5.1A) and secondly the same in vitro ARF1 guanine nucleotide exchange activity i.e. the same specific activity (Figure 5.1B). As shown in Figure 5A, the fully myristoylated ARF1 had very little effect on PLD activation at concentrations < 100 nM in the presence of 1 mM GTP. The use of GTP in these reconstitution assays is clearly a more physiologically relevant situation than one using GTPγS. This is because a single G-protein targeted by the GEFs plus GTP allows a more specific investigation of PLD activation without the indiscriminate co-activation of other G-proteins that will occur with GTPγS present. The choice of 1 mM GTP is based on the fact that this concentration is thought to be in the upper-end of the range of estimated levels of GTP in cells. Thus the basal activation of PLD was observed at 300 nM ARF1 and was slightly increased at 1 μM ARF1 stimulated by 1 mM GTP. The stimulation of PLD by 300 nM ARF1 was then significantly enhanced by ARNO (P = 0.04, n = 5), GRP-1 (P = 0.004, n = 5) and cytohesin-1 (P = 0.004, n = 5). The highest PLD activity was obtained at 1 μM ARF1 and in turn this was further improved by GRP-1 (P = 0.003, n = 5) and ARNO (P = 0.02, n = 5). Intriguingly, cytohesin-1 caused a slight inhibition of PLD activity at 1 μM ARF1 since the effects of cytohesin-1 on PLD activity was not significantly
Figure 5.1. Effects of GEFs on the activation of PLD by myristoylated ARF1 in permeabilized HL-60 cells. After 10 min permeabilization with SLO (0.4 IU/mL) at 37°C, [3H]choline-labelled and cytosol-depleted HL-60 cells were centrifuged and resuspended in buffer containing 3 mM MgATP and 3 mM Ca²⁺/EGTA (pCa5). The cells were then incubated for 20 min at 37°C with increasing concentrations of myristoylated recombinant ARF1 in the presence 1 mM GTP. (A) GRP-1, ARNO and cytohesin-1 normalised to the same concentration and (B) or to the same exchange activity were added to the reaction mixture. The assay was then performed as described in the Materials and Methods section. Data shown are mean ± S.E.M. of duplicate samples from 5 experiments. The significance of data was evaluated using Student t test.
different from that of GTP alone \( (P = 0.60, n = 5) \). However, there was no significant difference between the effect of cytohesin-1 on PLD activity obtained at 300 nM ARF1 and that obtained at 1 µM ARF1 \( (P = 0.40, n = 5) \). Interestingly, GRP-1 \( (P = 0.03, n = 5) \) and ARNO \( (P = 0.04, n = 5) \) exerted a significantly stronger effect on PLD activity than cytohesin-1 when 1 µM ARF1 was used in conjunction with GTP.

My earlier data showed that the small ARF-GEFs did not have the same degree of efficiency in their ability to exchange GDP for GTP on ARF1 when assayed in \textit{in vitro}, liposome-based nucleotide exchange assays. Indeed, my earlier data showed that ARNO was the most potent exchange factor on ARF1, followed by GRP-1 and then cytohesin-1. However, this order of potency varied from batch to batch of the different GEFs and it was not systematically observed. Sometimes GRP-1 and cytohesin-1 were also found to be equally efficient in their exchange activity on ARF1 or in some cases GRP-1 turned out to be the most efficient GEF. Therefore, in order to take account of this variability, the amount of each GEF used in the experiments was normalised to \textit{in vitro} exchange activity (Figure 5.1B). Under these specified experimental conditions, the activation of PLD detected at 300 nM ARF1 was again significantly improved by GRP-1 \( (P = 0.008, n = 5) \), ARNO \( (P = 0.02, n = 5) \) and cytohesin-1 \( (P = 0.01, n = 5) \). Again, the strongest PLD activity was observed at 1 µM ARF1 and further enhanced by ARNO \( (P = 0.02, n = 5) \) and GRP-1 \( (P = 0.03, n = 5) \). Again, cytohesin-1 apparently became slightly inhibitory at 1 µM ARF1 although this effect was not significantly different from that obtained at 300 nM ARF1 \( (P = 0.64, n = 5) \). In addition, the GEFs displayed no significant difference in their ability to enhance the ARF-mediated PLD activation when 1 µM ARF1 was used \( (P > 0.05, n = 5) \).
Under both experimental conditions, all three recombinant GEFs significantly improved the stimulation of PLD activity by ARF1 over the whole range of ARF1 concentrations. However, the GEFs did not show any statistically significant difference in potency between each other over the whole range of ARF1 concentrations ($P > 0.05$, $n = 5$). Unexpectedly, cytohesin-1 seemed to show an inhibitory effect at high ARF1 concentrations regardless of whether the GEF concentration was normalised to protein mass or exchange activity and the effect of cytohesin-1 on PLD activation reached a plateau at 1 μM ARF1. Whether this apparent but not conclusive inhibitory effect of cytohesin-1 on PLD activity is physiologically relevant is not clear.

In contrast to the results obtained in the reconstitution system using permeabilized HL-60 cells, the studied GEFs did not substantiate the stimulatory effects induced by either ARF1 or ARF6 on PLD activity in a cell-free system (Figure 5.2). The data shown in this figure clearly confirmed again what was already described in Chapter 3, that ARF1 is a stronger activator of PLD than ARF6 in vitro. Furthermore, increasing the concentration of GTP did not improve the activation of PLD by ARF1 in the presence of GEF, cytohesin-1 is given as an example in Figure 5.3.

The observation that ARNO, GRP-1 and cytohesin-1 failed to enhance the activation of PLD by ARFs in vitro could be explained by the fact that the GEF might have difficulty interacting with ARF and PLD under the specified experimental conditions. In particular, the purified GST-hPLD1b was recovered on glutathione sepharose beads. The activity of the immobilised enzyme was then assayed in these
Figure 5.2. Effects of GEFs on in vitro PLD activation mediated by myristoylated ARF1 and ARF6. Sf9 cells were infected with recombinant baculovirus for the expression of hPLD1b which was recovered on glutathione sepharose beads. A sample of the hPLD1b on beads (20 μl) was incubated with a fixed concentration of mARF1 (500 nM) or 238 nM mARF6 used with 1 mM GTP, 200 nM mARF6 used 1 μM ARNO, 238 nM mARF6 used with 1 μM GRP-1, 313 nM mARF6 used with 1 μM cytohesin-1 at 37°C for 60 min in the presence of PC substrate (8.6 μM final concentration) and [3H]choline-labelled PC (200000 dpm per 10 μl substrate). The different ARF6 concentrations used in this particular assay were determined based on the normalization of its exchange activity compared to mARF1. Reactions were stopped by transferring cells to ice and by adding methanol/chloroform (1:1, v/v). Phases were partitioned by addition of water. Release of [3H]choline was measured as described in the Materials and Methods section. Data shown are mean ± S.E.M. of duplicate samples from 3 experiments. The significance of data was evaluated using Student’s t test.
Figure 5.3. In vitro PLD activation mediated by myristoylated ARF1: effects of cytohesin-1 and various concentrations of GTP. Sf9 cells were infected with recombinant baculovirus for the expression of hPLD1b which was recovered on glutathione sepharose beads. A sample of the hPLD1b on beads (20 µl) was incubated with a fixed concentration of 500 nM mARF1 at 37°C for 60 min in the presence of PC substrate (8.6 µM final concentration) and [3H]Choline-labelled PC (200000 dpm per 10 µl substrate). GTP was added to the reaction mixtures at the indicated concentrations and along with 10 µM cytohesin-1. Reactions were stopped by transferring cells to ice and by adding methanol/chloroform (1:1, v/v). Phases were partitioned by addition of water. Release of [3H]choline was measured as described in the Materials and Methods section. Data shown are mean ± S.E.M. of duplicate samples from 3 experiments. The significance of data was evaluated using Student's t test.
experiments. This procedure might conceivably hinder the access of ARF and cytohesin-1 to PLD. To test this hypothesis, reduced glutathione was included in the assay in order to release PLD in the presence of ARF1 and cytohesin-1. As shown in Figure 5.4, treatment of PLD beads with 2.5 mM GSH improved the activation of PLD by ARF1 plus GTP. The PLD activity was significantly higher after treatment with 2.5 mM GSH compared to 10 mM GSH (P = 0.02, n = 3) or to the control (P = 0.03, n = 3). However, cytohesin-1 did not potentiate the stimulatory effect on PLD activity induced by ARF1 plus GTP, even though the treatment of PLD beads with 2.5 mM GSH prior to PLD activation did significantly increase PLD activity. This effect of 2.5 mM GSH in improving PLD activity was again significantly stronger than that obtained with 10 mM GSH (P = 0.02, n = 3) or the control (P = 0.02, n = 3). Thus the results illustrated in Figure 5.3 clearly indicate that the failure of cytohesin-1 to enhance PLD activity stimulated by ARF1 cannot be attributed to the fact that the use of glutathione sepharose beads to recover and then present PLD might have impeded the access of the regulatory proteins to PLD.

5.2.2. Negative effects of GTP on the stimulation of PLD activity through the ARF-GEF pathway

The effects of increasing concentrations of GTP were examined in permeabilized HL-60 cells. As shown in Figure 5.5, cytohesin-1 alone had no effect on PLD activity over the whole range of GTP concentrations. Interestingly, and not too surprisingly, given results described in Chapter 3, ARF1-alone could induce an activation of PLD in the absence of GTP. This increase in PLD activity was significant (P = 0.007, n = 4) compared to the control. The most intriguing observation was the ability of
Figure 5.4. Effects of pretreatment of PLD1b on glutathione sepharose beads with GSH. The activity of recombinant PLD1b purified from sf9 insect cells transfected with the hPLD1b baculovirus was determined either in the absence of GSH or in the presence of 2.5 mM GSH and 10 mM GSH. Prior to incubation of PLD1b with recombinant ARF1 (500 nM) and/or cytohesin-1 (10 μM), in the presence of GTP (1 mM), a sample of the purified PLD1b recovered on glutathione sepharose beads was treated with the indicated concentrations of GSH for 30 min at 4°C. Pretreated PLD1b was then incubated with substrate prepared from PE, PIP₂ and PC (C₁₀) in the molar ratio 10: 0.3: 1. The final PC concentration was 8.6 μM. [³H]Choline-labelled didecanoyl PC was added to give approximately 200,000 d.p.m. per 10 μL substrate. The reaction mixtures were transferred to a 37°C water bath and incubated for 1 hour. Release of [³H]Choline was measured as described in the Materials and Methods section. Data shown are mean ± S.E.M. of duplicate samples from 3 experiments.
Figure 5.5. Negative effects of GTP on the activation of PLD by fully myristoylated ARF1 and cytohesin-1 in permeabilized HL-60 cells. After 10 min permeabilization with SLO (0.4 IU/mL) at 37°C, [3H]choline-labelled and cytosol-depleted HL-60 cells were centrifuged and resuspended in buffer containing 3 mM MgATP and 3 mM Ca\(^{2+}\)/EGTA (pCa5). The cells were then incubated for 20 min at 37°C with increasing concentrations of GTP (lithium salt) in the presence or absence of the indicated concentrations of myristoylated ARF1 and cytohesin-1. The assay was then terminated as described in the Materials and Methods section. Data shown are mean ± S.E.M. of duplicate samples from 4 experiments.
cytohesin-1 to significantly enhance ARF1-mediated PLD activation without the requirement for exogenous GTP. Indeed, PLD was activated by the combined action of ARF1 and cytohesin-1 in the absence of any added GTP leading to 0.36% of total lipids being hydrolyzed. This effect was significantly higher \( (P = 0.02, n = 4) \) than the one obtained in the presence of ARF alone which induced 0.22% of total lipids to be hydrolyzed. The observed ARF1-stimulated-PLD activity enhanced by cytohesin-1 in the absence of exogenous GTP could be due to a preferred pool, or to other source of endogenous GTP that cytohesin-1 could use to drive ARF1 into its active conformation. It is also possible that a nucleoside diphosphate kinase (NDPK) catalyzes the phosphorylation of endogenous GDP molecules either free in solution or bound to ARFs (Randazzo et al., 1992; Randazzo et al., 1991; Kikkawa et al., 1990).

In addition, it was also found that adding increasing concentrations of exogenous GTP gradually decreased the stimulatory effect of cytohesin-1 on ARF1-stimulated PLD activity. However, even at high concentrations of GTP such as 1 mM GTP, cytohesin-1 could still significantly enhance PLD activity \( (P = 0.02, n = 4) \) as already observed in Figure 5.1. But at GTP concentrations > 1 mM, the stimulatory effect of cytohesin-1 was completely abolished. In summary, it is not clear at this stage if a guanine nucleotide is an absolute requirement for PLD activation in HL-60 cells and whether, if one is required, if it need be GTP.

5.2.3. The ARF1-stimulated PLD activity that is enhanced by small ARF-GEFs preferentially uses GDP over exogenous GTP as a source of guanine nucleotide

As described above, the activation of PLD through the ARF-GEF pathway does not seem to rely on bulk, soluble GTP for ARF activation. Bearing this in mind, one
hypothesis is that the system can operate effectively using GDP. The following experiments were performed to test this hypothesis. Firstly, a range of concentrations of GEFs were tested for their ability to enhance the activation of PLD by ARF1 in the presence and in the absence of GTP in permeabilized HL-60 cells. After cell permeabilization, the cells were resuspended in buffer containing a final concentration of 100 μM GDP. As shown in Figure 5.6A, the activation of PLD by cytohesin-1 is concentration-dependent. Cytohesin-1 alone or combined with exogenous GTP did not have any effect on PLD activity. Interestingly, cytohesin-1 could significantly improve the ARF1-stimulated PLD activity by two-fold at concentrations as low as 400 nM (P = 0.002, n = 3) compared to the PLD activity stimulated by ARF1 alone. Encouragingly, this GTP-independent effect was dependent on the concentration of cytohesin-1. There is no significant difference between the basal PLD activity (control or GTP alone) and that observed in the presence of ARF alone or ARF plus GTP in the absence of cytohesin-1. The ARF1-stimulated PLD activity seemed to be maximal between 12 and 40 μM cytohesin-1 where a significant three-fold increase was observed compared to the PLD activity stimulated by ARF1 alone (P = 0.007, n = 3). However, the enhancement of the ARF1-stimulated PLD activity by cytohesin-1 observed in the presence of the exogenously added GTP was not statistically significant (P > 0.05, n = 3) over the whole range of concentrations tested, if they were compared to the PLD response induced by ARF1 plus GTP in the absence of cytohesin-1. A clear feature stands out: the improvement of the ARF1-stimulated PLD activity by cytohesin-1 was significantly greater in the absence of added GTP than in the presence of added GTP (P < 0.05, n = 3). This was true for the concentrations of
Figure 5.6. Concentration dependence of GEF-induced PLD activation in permeabilized HL-60 cells: negative effects of GTP. After permeabilization, cells were centrifuged and resuspended in buffer containing 3 mM MgATP and 3 mM Ca\(^{2+}/\text{EGTA (pCa}5)\) and 100 μM GDP. The cells were then incubated for 20 min at 37°C with increasing concentrations of (A) cytohesin-1, (B) GRP-1, (C) ARNO in the presence or absence of fully myristoylated ARF1 and GTP as indicated. The assay was then performed as described in the Materials and Methods section. Data shown are mean ± S.E.M. of duplicate samples from 3 experiments for cytohesin-1 concentration-dependence effects.
cytohesin-1 ranging from 0.1 μM to 40 μM. It is interesting to note that at high concentrations, cytohesin-1 showed a small inhibitory effect in the presence of exogenous GTP. This effect was already noted in Figure 5.1.

Similarly, GRP-1 and ARNO also showed the same stimulatory effect on PLD activity as cytohesin-1. This effect was once again more potent in the absence of GTP at concentrations of GEFs > 1 μM. GRP-1 barely improved the activation of PLD by ARF1 over the whole range of concentrations tested in the presence of GTP (Figure 5.6B) whereas ARNO displayed a slightly more potent stimulatory effect on PLD activity in the presence of GTP (Figure 5.6C).

Since the results shown above seem to strongly indicate a positive effect of GDP in activating PLD via the ARF-GEF pathway, a range of GDP concentrations was tested (Figure 5.7A). Using permeabilised cells and by simply adding ARF1, with or without GTP, to cytohesin-1-containing reactions devoid of added GDP produced a statistically significant increase in the basal PLD activity of about three fold. GDP alone or in the presence of 40 μM cytohesin-1 with or without the added GTP did not elicit a PLD response. However, concentrations of GDP between 10 μM and 100 μM significantly increased PLD activation by ARF1 and cytohesin-1 (P < 0.05, n = 3). At these concentrations of GDP, the addition of 1 mM GTP decreased PLD activity by half. At concentrations > 100 μM, the effects of GDP became inhibitory. Conceivably this inhibition by higher GDP concentrations could result from competition with endogenous GTP, present in the cells, for ARF. Thus the optimal GDP concentration found was 100 μM. This concentration was used in other experiments where a range of concentrations of cytohesin-1 was tested in the presence of ARF1 with or without
Figure 5.7. The activation of PLD in permeabilized HL-60 cells mediated by ARF1 and cytohesin-1 is dependent on GDP and is not inhibited by PAPS, an NDPK inhibitor. (A) Concentration dependence of GDP-induced activation of PLD, (B) concentration dependence of cytohesin-1 and (C) effects of PAPS on the stimulation of PLD activity by ARF1 and cytohesin-1 in the presence and absence of GTP. The standard PLD assay in permeabilized HL-60 cells was performed as described in the Materials and Methods section. 100 μM GDP was added to the resuspended HL-60 cells after permeabilization. Data shown are mean ± S.E.M. of duplicate samples from 3 experiments.
added GDP. As shown in Figure 5.7B, cytohesin-1 alone or combined with added GDP was not effective over the whole range of the indicated concentrations. Upon addition of 300 nM myristoylated ARF1 in the presence of GDP, cytohesin-1 gradually increased its stimulatory effect on PLD activity with increasing concentration. The maximal effective concentration of cytohesin-1 was 40 μM. This observed effect of cytohesin-1 can only be attributed to the presence of GDP since in its absence the activation of PLD by ARF1 was not observed over the whole range of cytohesin-1 concentrations. Indeed, concentrations of cytohesin-1 greater than 1 μM were required to reveal a GDP-dependent PLD response to ARF1 (P < 0.05, n = 3).

5.2.4. The NDPK competitive inhibitor, PAPS, has no effect on the GDP-sensitive activation of PLD by ARF1

The experiments described in Figures 5.7A and 5.7B clearly show a critical role for GDP in mediating the activation of PLD through the small ARF-GEF pathway. One possible explanation for this finding could be a role for NDPK. This enzyme converts GDP into GTP and was shown to be active in a related experimental system, permeabilized mast cells (Martin et al., 1995). However, the use of PAPS as a competitive inhibitor of the NDPK did not decrease the stimulatory effect of GDP (Figure 5.7C). The PLD response to ARF1 and cytohesin-1 with or without the added GTP was significantly higher than the basal PLD activity obtained in response to cytohesin-1 alone or in the presence of GTP. The lack of significant difference in PLD activation caused by ARF1 and cytohesin-1 in the absence and presence of GTP at low PAPS concentration may be artefactual. For the experiments shown in Figures 5.7A and 5.7B clearly demonstrated that the PLD activity stimulated by ARF1 and
cytohesin-1 was maximal in the presence of 100 μM exogenously added GDP and decreased by half when GTP was added to the reaction mixtures. As PAPS was titrated in the reaction mixtures, PLD activity induced by ARF1 and cytohesin-1 remained unchanged. One would expect PAPS to inhibit the production of endogenous GTP from the added GDP catalysed by the NDPK. PAPS has been shown to bind NDPK with a $K_D$ of 10 μM in in vitro studies using a NDPK kinase from Dictyostelium amoeba (Schneider et al., 1998).

Although the activation of PLD is GDP-sensitive in the permeabilized HL-60 cell system, presumably due to the presence of a system responsible for the conversion of GDP into GTP, the addition of GDP in cell-free in vitro PLD assays containing recombinant PLD, ARF1 and cytohesin-1 failed to stimulate PLD (Figure 5.8). In the same fashion, this can only be attributed to the lack of that mechanism, present in the cells, that allows GDP-dependent activation. The unidentified mechanism was obviously absent in the cell-free system where only GTP was effective in activating PLD in vitro.

These data seem to support the hypothesis that GDP is a better source of guanine nucleotide for ARF activation through the small GEFs in permeabilized HL-60 cells. Taking advantage of such unexpected stimulatory effects of GDP, it was therefore reasonable to introduce a fourth element in the so far defined PLD signalling pathway comprising of GEFs, ARFs and PLD. This fourth element is either PIP$_2$ or PIP$_3$. These two polyphosphoinositide have already been shown to participate in the ARF-regulated PLD activity mostly in vitro (Hammond et al., 1997; Hammond et al., 1995; Pertile et al., 1995; Liscovitch et al., 1994; Brown et al., 1993).
Figure 5.8. *In vitro* PLD activation mediated by myristoylated ARF1: comparison of the effects GTP and GDP. Sf9 cells were infected with recombinant baculovirus for the expression of hPLD1b which was recovered on glutathione sepharose beads. A sample of the hPLD1b on beads (20 μl) was incubated with a fixed concentration of 500 nM mARF1 at 37°C for 60 min in the presence of PC substrate (8.6 μM final concentration) and [3H]Choline-labelled PC (200000 dpm per 10 μl substrate). GTP (100 μM) and GDP (100 μM) was added along with 10 μM cytohesin-1. Reactions were stopped by transferring cells to ice and by adding methanol/chloroform (1:1, v/v). Phases were partitioned by addition of water. Release of [3H]choline was measured as described in the Materials and Methods section. Data shown are mean ± S.E.M. of duplicate samples from 3 experiments. The significance of data was evaluated using Student’s *t* test.
5.2.5. Both PIP₂ and PIP₃ enhance PLD activity in HL-60 cells and in vitro but PIP₃ is a better PLD activator

The effects of further additions of exogenous PIP₂ and PIP₃ on the coupling between GEFs, ARFs and PLD were investigated in cell-based and cell-free systems. As illustrated in Figure 5.9, under the “control” condition, the addition of recombinant cytohesin-1 and PIP₂ and PIP₃ slightly augmented the basal PLD activity in HL-60 cells. This small increase in PLD responsiveness to cytohesin-1 and the polyphosphoinositides was significant with PIP₃ (P = 0.03, n = 3) but not with PIP₂ (P = 0.06, n = 3). Under the “plus mARF” condition, the subsequent addition of myristoylated ARF1 further enhanced the PLD basal activity found under the “control” condition. Not too surprisingly, PLD responsiveness to ARF1 alone was significantly increased by the addition of cytohesin-1 alone (P = 0.01, n = 3), cytohesin-1 plus PIP₂ (P = 0.006, n = 3), and cytohesin-1 plus PIP₃ (P = 0.01, n = 3). More interestingly, the further enhancement of PLD activity induced by the polyphosphoinositides in response to ARF1 plus cytohesin-1 was significant with PIP₃ (P = 0.03, n = 3) but not with PIP₂ (P = 0.1, n = 3) although there is no significant difference between the enhancing effect of these two polyphosphoinositides in comparison to the PLD activity elicited by ARF1 plus cytohesin-1 (P = 0.1, n = 3). Similar to the “control” condition, under the “plus GTP” condition, PIP₃ appeared to be the most effective activator of PLD as it significantly increased the basal PLD response (P = 0.02, n = 3). Under the “mARF1 + GTP” condition, the presence of GTP did not affect the potentiating effect of either cytohesin-1 alone or cytohesin-1 plus PIP₂/PIP₃. Indeed, under the “mARF1 + GTP” condition, the basal PLD activity
Figure 5.9. Effects of PIP$_2$ and PIP$_3$ on PLD activity in permeabilized HL-60 cells. Activity of PLD was determined under standard assay conditions in previously permeabilized HL-60 cells in the presence or absence of 500 nM ARF1, 1 μM cytohesin-1, 30 μM C$_8$ PIP$_2$, 30 μM C$_8$ PIP$_3$, 100 μM GTP as indicated in the legend. After permeabilization, HL-60 cells were centrifuged and resuspended in buffer containing 3 mM MgATP and 3 mM Ca$^{2+}$/EGTA (pCa5) and 100 μM GDP. Release of [³H]Choline was measured as described in the Materials and Methods section. Data shown are mean ± S.E.M. of duplicate samples from 3 experiments. The significance of data was evaluated using Student $t$ test.
rose significantly when cytohesin-1 alone (P = 0.03, n = 3), cytohesin-1 plus PIP$_2$ (P = 0.007, n = 3), and cytohesin-1 plus PIP$_3$ (P = 0.002, n = 3) were present. Although no significant difference was observed between the effects of cytohesin-1 alone and cytohesin-1 plus PIP$_2$, cytohesin-1 plus PIP$_3$ significantly yielded stronger enhancing effects than those produced by cytohesin-1 alone (P = 0.01, n = 3) and cytohesin-1 plus PIP$_2$ (P = 0.0008, n = 3). Clearly, the results found in HL-60 cells indicate that the activation of PLD by the combined actions of ARFs and GEFs is undoubtedly further increased by the exogenous addition of polyphosphoinositides such as PIP$_2$ and PIP$_3$. However, PIP$_3$ turns out to be the most effective activator of PLD under these conditions. This observation was backed up to some extent by in vitro assays, again the exogenous effects of PIP$_2$ and PIP$_3$ on recombinant GST-hPLD1b activity stimulated by ARF1 plus GTP.

Indeed, as shown in Figure 5.10, the basal activity of PLD was substantially elevated in the presence of PIP$_2$ and PIP$_3$. PIP$_3$ had a stronger effect than PIP$_2$. In the presence of ARF1 plus GTP, the basal activity of PLD was stimulated approximately 40-fold. This increase was further potentiated by the addition of PIP$_2$ and PIP$_3$. Again, PIP$_3$ produced a stronger activation of PLD mediated by ARF1 and GTP than PIP$_2$. Cytohesin-1 in combination with GTP did not trigger a marked PLD response although the basal PLD activity seemed to be slightly improved. However, cytohesin-1 did not enhance the GTP plus ARF1-stimulated activity of PLD either in the presence or absence of PIP$_2$ and PIP$_3$. It is conceivable that in this in vitro PLD assay, cytohesin-1 sequesters some of the exogenously added PIP$_2$ and PIP$_3$ thereby preventing these phosphoinositides from fully activating PLD.
Figure 5.10. Effects of PIP$_2$ and PIP$_3$ on the activity of recombinant PLD1b purified from sf9 insect cells transfected with the hPLD1b baculovirus. A sample of the purified PLD1b recovered on glutathione sepharose beads previously treated with 2.5 mM GSH for 30 min at 4°C was incubated with substrate prepared from PE, PIP$_2$ and PC (C$_{10}$) in the molar ratio 10: 0.3: 1. The final PC concentration was 8.6 μM. [$^3$H]Choline-labelled didecanoyl PC was added to give approximately 200, 000 d.p.m. per 10 μL substrate. Recombinant ARF1 (500 nM), cytohesin-1 (10 μM), C$_8$ PIP$_2$ (10 μM) and C$_8$ PIP$_3$ (10 μM) were added to the reaction mixture in the presence of GTP (1 mM). The reaction mixtures were transferred to a 37°C water bath and incubated for 1 hour. Release of [$^3$H]Choline was measured as described in the Materials and Methods section. Data are representative of two experiments performed in duplicates.
5.3. Discussion

This thesis is the first to examine the properties of the reconstitution of a ternary complex consisting of GEFs, ARFs, and PLD in both permeabilized HL-60 cells and in cell-free assays. In permeabilized HL-60 cells, PLD activation induced by ARF1 was significantly promoted by the presence of either ARNO, GRP-1 or cytohesin-1. The nucleotide used as an activator of ARF was GTP. The concentration of GTP used in the majority of reconstitution assays was 1 mM. Even at this high concentration of GTP, the GEFs were still able to significantly stimulate the ARF1-mediated PLD activity with the same degree of potency. The effects of these GEFs on PLD activity in HL-60 cells were verified through the normalization to protein concentration and to the exchange specific activity. Under both experimental conditions, all three of the GEFs tested were equally potent. The equipotency of the GEFs was observed at relatively low concentrations of ARF1 (300 nM). A minimum of 100 nM ARF1 was required to observe any stimulation with the GEFs. ARNO and GRP-1 were still approximately equipotent, at higher concentrations, stimulating maximally at 1 μM ARF1. However, cytohesin-1 caused a slight inhibition of PLD activity. This effect may be explained by the fact that at this particular concentration, the activation of ARF1 by cytohesin-1 could be accompanied by the activation of inhibitory pathways. By contrast, the same GEFs were unable to enhance the activation of PLD by ARF1 or ARF6 in vitro using a recombinant PLD1b purified from sf9 insect cells transfected with a hPLD1b-coding baculovirus and recovered on glutathione sepharose beads. This observation underlines major differences between the two model systems described in this thesis. Indeed, it seems very likely that cells possess other specific
and/or non-specific components that cell-free systems do not necessarily have, even though rudimentary efforts have been made to mimic the "natural" environment of the cell.

The specific components could be specific protein activators and essential lipids. Protein activators could be residual members of the small G protein family (i.e. Rho and Ral family proteins), PKC or other cytosolic factors present in the cells after permeabilization. These proteins may act in concert with ARFs and GEFs to bring about an effective and optimal activation of PLD in HL-60 cells. For example, the membrane-associated RhoA, a member of the Rho family of small G-proteins, and ARF were shown to synergistically activate PLD in HL-60 membranes (Siddiqi et al., 1995) and partially purified rat brain PLD (Kuribara et al., 1995) in the presence of GTPγS. Interestingly, Singer et al. (1996) reported that PLD in porcine brain was synergistically activated in vitro by PKC, ARF and RhoA in the presence of GTPγS. Similarly, a combination of PKCα, ARF1 and Cdc42 significantly stimulated PLD activity in the detergent-insoluble fraction of HL-60 membranes (Hodgkin et al., 1999). Another member of the small G protein, RalA, originally shown to directly interact with PLD1 (Luo et al., 1997), has been reported to synergistically stimulate bovine brain PLD activity in concert with ARF1 in vitro (Kim et al., 1998). Recently, Powner et al. (2002) have provided in vivo evidence for a co-localization of PLD1b with Rac1, ARF6, and PKCα in actin-rich structures at the plasma membranes following antigen-stimulation of RBL-2H3 cells. Furthermore, a 50 kDa cytosolic factor in combination with either purified bovine brain ARF1 or ARF3 or purified
recombinant human ARF1 was able to synergistically activate PLD in human peripheral blood neutrophils (Lambeth et al., 1995).

Thus it appears that the normal activation of PLD is multifactorial and therefore it is likely that in a cellular environment those reported regulatory molecules would conspire to promote a complete activation of PLD. However, the precise mechanism by which ARF synergizes with these co-regulatory molecules in cells remains unclear. Nonetheless, most in vitro studies seem to suggest that the mechanism of the synergistic activation of PLD is mediated by the direct and independent interaction of the PLD-activating factors with PLD (Kim et al., 1998; Lee et al., 1997).

As already discussed in the previous chapter, phospholipids are closely involved in ARF and GEF functions. In the in vitro PLD assay described in this thesis, the substrate was prepared from a mixture of PIP$_2$, and neutral phospholipids such as PC and PE. However, acidic lipids such as PS and PG might also be critical in the cellular environment. In addition to the critical role of phosphoinositides, these acidic phospholipids might contribute to the optimal non-specific interaction between the GEFs and the cellular membranes to which they are recruited following receptor stimulation. Indeed, in vitro studies demonstrated, for instance, that the stimulation by ARNO of [$^{35}$S]GTPyS binding on ARF1 in guanine nucleotide exchange assays and binding of ARNO to lipid vesicles in sedimentation experiments were optimal under conditions where the PC vesicles were supplemented with either PG or PS in addition to PIP$_2$ (Macia et al., 2000; Chardin et al., 1996). Each of these acidic phospholipids has a single negative charge that would presumably enhance the binding of the GEFs to phosphoinositides in membranes, a process mediated by their positively charged PH
domains, in a cellular context. The composition of PS and PG is thought to be up to around 30% of the phospholipids on the cytoplasmic leaflets of cellular membranes (Macia et al., 2000).

The permeabilization “model system” allows the leakage of cytosolic components out of the perforated cells but the integrity of the non-specific components such as membranes of cellular organelles including the nucleus, ER, Golgi apparatus, transport/secretory vesicles and plasma membrane generally remain intact when analysed by electron microscopy (Geraint Thomas, unpublished observation). The structural features of these membranes such as their curvature and their lipid and protein composition are implicated in cellular functions involving internalization processes including endocytosis (Hurley and Wendland, 2002; Nossal and Zimmerberg, 2002), podocytosis (Anderson et al., 1992) and transcytosis (Ghitescu et al., 1986). For instance, PLD activity has been found in specific microdomains of the plasma membranes identified as caveolae or lipid rafts (Severs, 1988; Harder and Simons, 1997). These small vesicular invaginations are present in most cell types (Anderson, 1998; Parton, 1996) and have been suggested to be involved in the regulation of PLD activity because they are enriched in critical PLD-regulatory molecules such as PIP\(_2\) (Hope and Pike, 1996; Pike and Casey, 1996), heterotrimeric GTP-binding proteins (Chang et al., 1994; Lisanti et al., 1994), the Ras-related GTP-binding proteins (Gingras et al., 1998; Senda et al., 1997; Chang et al., 1994; Lisanti et al., 1994) and PKC (Kim et al., 2000; Kim et al., 1999). It has been suggested the PLD activity detected in caveolae was not PLD1 but PLD2 (Czarny et al., 2000; Czarny et al., 1999). However, this observation was restricted to cell types
including HaCaT human keratinocytes and U937 promonocytes. Although there is no evidence for an ARF-activated PLD in caveolae in HL-60 cells, one cannot exclude the existence of a phosphoinositide-dependent GEF-ARF-PLD pathway in the caveolar structures since these microdomains harbour essential signalling molecules required for PLD activity.

The specific and non-specific features evoked above are intrinsic components of a cellular environment that are difficult to mimick in vitro and the lack of some of these described cellular components in the cell-free assay might therefore explain the failure of the GEFs to effectively potentiate the ARF-mediated activation of PLD observed in the permeabilized model system. For example, my in vitro assays with recombinant PLD1b cannot reproduce the caveolar micro environment.

As observed in permeabilized HL-60 cells, the GEFs were able to increase PLD responsiveness to ARF1. The initial failure to detect such stimulatory effects in vitro may be attributed to sub-optimal levels of essential phosphoinositides such as PIP$_2$ and PIP$_3$ in the reaction mixtures. As a result, exogenous PIP$_2$ and PIP$_3$ were added to the recombinant PLD1b in the presence of recombinant ARF1. The in vitro experiment illustrated in Figure 5.10 clearly demonstrated that both basal and ARF1-stimulated PLD activity was mostly enhanced by the addition of exogenous phosphoinositides such as PIP$_2$ and PIP$_3$. The addition of cytohesin-1 had no further effects. However, in a cellular context, the same cytohesin-1 significantly augmented the activation of PLD by GTP-activated ARF1. Of similar interest is the observation that PIP$_2$ and PIP$_3$ further potentiated the stimulatory effects of cytohesin-1 on ARF1-stimulated PLD activity when assayed in permeabilised cells. However, the observed higher potency
displayed by PIP₃ in permeabilized cells and *in vitro* assays seems to contradict earlier studies showing that PIP₂ was somewhat more effective than PIP₃ in activating PLD (Hammond *et al*., 1997; Liscovitch *et al*., 1994). But overall, these results are in agreement with previous reports on the requirement for polyphosphoinositides in ARF-regulated PLD activity. Indeed, The PIP₂-dependent activation of PLD by ARF is well-established both *in vitro* (Liscovitch *et al*., 1994; Massenburg *et al*., 1994; Brown *et al*., 1993) and in permeabilized cells (Schmidt *et al*., 1996; Pertile *et al*., 1995). The role of PIP₃ in elevating PLD activity has also been reported *in vitro* (Hammond *et al*., 1997; Liscovitch *et al*., 1994). The fact that exogenous PIP₃ can participate in PLD activation in permeabilized HL-60 cells and *in vitro* further supports a possible role for the PI 3-kinase pathway in the activation of PLD by agonists which cause transient increases in the levels of PIP₃ in cells. However, the mechanism by which these phosphoinositides activate PLD, or rather increase the activation of PLD stimulated by ARF1 *in vitro*, is still unclear. It is possible that the presence of either PIP₂ or PIP₃ increases the binding affinity of the purified PLD to lipid vesicles via the proteins PX domain. Alternatively, either of these two phosphoinositides may alter the substrate-containing phospholipid surface in a way that renders the PC substrate more accessible for the enzyme. Interestingly, the observed increase of the basal activity of PLD induced by PIP₂ and PIP₃ might indicate a direct interaction of PLD with the phosphoinositides. Indeed, a short conserved sequence (21 residues) containing hydrophobic amino acids interspersed with basic arginine and lysine residues has been identified as a PIP₂-binding site in mammalian PLD2 (Sciorra *et al*., 1999). This binding motif was also shown to be
present in mammalian PLD1 and the yeast PLD, Spo14p. However, the identified phosphoinositide-binding motif turned out to be absent from PLD enzymes that are not dependent on PIP2 for activity, such as bacterial PLD (Sciorra et al., 1999; Morris et al., 1996; Ponting and Kerr, 1996). Mutagenesis of the first two conserved arginine residues of the phosphoinositide-binding sequence in PLD2 significantly attenuated the catalytic activity of the mutants as well as their binding to PIP2-containing vesicles in vitro. The decrease in PLD activity was also observed in vivo (Sciorra et al., 1999).

Based on their published data and those of others (Chaudhary et al., 1998; Martin, 1998; Lu and Chen, 1997), Sciorra et al. (1999) suggested that the direct interaction between the identified phosphoinositide-binding motif in PLD and PIP2 is mediated by the conserved arginine and lysine residues that form electrostatic interactions with the phosphate groups on the inositol ring of PIP2. Other phosphoinositide-binding domains such as the PH and PX domains are present at the N terminus of PLD enzymes and these domains may also serve to bind either PIP2 or PIP3. However, the precise function of the PX and PH domains in PLD is currently not well-defined although the PH domain has been shown to play no significant role in the activation of PLD by PIP2 in vitro (Sciorra et al., 1999; Sung et al., 1999a; Sung et al., 1999b). On the other hand, it is conceivable that the mechanism of activation of PLD by PIP2 or PIP3 might be mediated through direct interaction of the phosphoinositides with ARF1. Thus the newly formed complex would then interact with PLD. Evidence for a direct in vitro interaction between PIP2 and ARF1 has been reported (Randazzo, 1997; Terui et al., 1994). PIP2 has been suggested to act as an exchange factor on ARF1 by stimulating the rate of GDP dissociation from ARF1 and stabilizing the nucleotide-
free form (Zheng et al., 1996; Terui et al., 1994). However, the role of PIP$_2$ in activating ARF is further complicated by the fact that it also potentially plays a role by recruiting ARF-GEF proteins to membranes.

More intriguing still is the fact that PIP$_3$ is unquestionably a better activator of PLD than PIP$_2$ under the two experimental conditions described in this thesis. What is the significance of such difference? It is well known that PIP$_2$ is a stable, constituent of several cell membranes, the levels of which are maintained at some steady concentration. Consequently this lipid is always available in cells to participate in PLD-signaling pathway (Skippen et al., 2002; Fensome et al., 1996), the PLC-signaling pathway (Berridge, 1993) and the PI 3-kinase-signaling pathway (Rameh and Cantley, 1999). By contrast, the appearance of significant amounts of PIP$_3$ is usually the result of the stimulation of cell surface receptors e.g. insulin receptors (Rizzo and Romero, 2002). Therefore the levels of PIP$_3$ fluctuates making its physiological actions transient as opposed to those of PIP$_2$. Interestingly, even in the aftermath of receptor stimulation, the levels of PIP$_3$ are still far smaller than those of PIP$_2$ (Czech, 2000) whose effective concentration in the cell is about 10 μM (McLaughlin et al., 2002). On the basis of these considerations, one puzzling question arises. What are the cellular mechanism(s) that render PIP$_3$ a more potent PLD activator than PIP$_2$? Is there one specific element in the multi-component PLD pathway that would preferentially use, for instance, PIP$_3$ as a better binding partner over PIP$_2$? Traditionally such a component is believed to be the PH domains of the ARF-GEFs. However, as already discussed in the previous chapter, I find that in contrast to other reports the GEFs cannot effectively discriminate between these two
phosphoinositides. This probably excludes the GEFs as a potential single discriminatory element.

Taken together, the data described in this thesis and those previously reported underline the possible mechanisms of activation of PLD by phosphoinositides. These phosphoinositides are likely to be multifunctional in their regulation of ARF-stimulated PLD activity.

As already discussed, ARF1 must ordinarily bind GTP to interact with target proteins such as PLD and this may reflect the need for translocation to membranes or specific interactions (see Chapter 3). Experiments in permeabilized HL-60 cells revealed inhibitory effects of GTP on PLD activity at concentrations in the millimolar ranges. This observation may be due to an activation of PIP$_2$ breakdown through the well-known PLC pathway. Indeed, it is possible that GTP or ATP activate a purinergic receptor coupled to a heterotrimeric G protein, which is supported by GTP, that is also present in these cells. The G protein might then activate the PLC pathway leading to a decrease in the levels of PIP$_2$ as a result of its breakdown by PLC. The products of this hydrolysis are the well-established second messengers, DAG and IP$_3$ (Berridge, 1993). The subsequent reduction of the levels of PIP$_2$ might decrease the responsiveness of PLD to ARF. On the other hand, it is plausible that the high concentrations of GTP support inhibitory enzymes. In addition, it is interesting to note that the form of GTP used in the experiments described in this chapter is the lithium salt. It has been suggested that lithium could interfere with G protein function by inhibiting GTP binding (Avissar et al., 1988).
The results presented in this chapter emphasize a critical and unexpected role for GDP in the activation of PLD by ARF1 and its exchange factors ARNO, GRP-1 and cytohesin-1 in HL-60 cells. This important result possibly implies a role of endogenously formed GTP which was preferentially effective in stimulating PLD activity compared to bulk exogenous GTP. Therefore an intracellular mechanism that converts either exogenous or endogenous GDP into GTP might be involved in the efficient activation of PLD by ARF1 in the presence of any of the GEFs studied. A potential candidate for this GTP-binding protein coupled effector regulation is the membrane-and-cytoskeleton-associated NDPK. NDPK was initially shown to catalyse the transfer of phosphate from ATP to GDP that had been previously found bound to ras (Ohtsuki and Yokoyama, 1987), ARF (Randazzo et al., 1991) and trimeric GTP binding proteins (Kikkawa et al., 1990), thereby possibly activating G proteins without the need for nucleotide exchange. However, the suggested direct interaction of these GTP-binding proteins with NDPK was subsequently dismissed on account of flaws in the experimental design (Randazzo et al., 1992). Evidence for the activity of NDPK in membranes of HL-60 cells has been published (Wieland and Jakobs, 1992, Wieland et al., 1991; Seifert et al., 1988). Based on these considerations, it is tempting to hypothesize that the observed GDP-dependent PLD activity in HL-60 cells would be the result of the conversion of GDP into GTP by NDPK. This hypothesis is further supported by the fact that the presence of ATP is required in the permeabilized cells model system described in this thesis. In addition to its role in maintaining the levels of intracellular PIP$_2$, ATP would serve as the phosphate donor in the conversion of GDP to GTP by NDPK. The newly synthesized GTP would then be used by the GEFs
to activate ARF. This mechanism would reconcile the previously proposed direct interaction between NDPK and GTP-binding proteins that was later called into question (Randazzo et al., 1992). In order to test this hypothesis, the effects of an inhibitor of NDPK were examined. Among various NDPK inhibitors employed in vitro and in permeabilized cells in previous studies (Schneider et al., 1998; Martin et al., 1995), PAPS is of special interest because it was shown to bind relatively well to the NDPK in vitro binding assays (Schneider et al., 1998). However, PAPS was without effect on the GDP stimulation of PLD in the model system described in this thesis. The failure of PAPS to inhibit NDPK and the presumed formation of endogenous GTP from GDP and ATP in HL-60 cells might indicate a low specificity of this inhibitor towards the particular type of NDPK present in this type of cells. Alternatively, other systems responsible for the synthesis of GTP might be operating in HL-60 cells.

Interestingly, the stimulatory effect of GDP was also observed in other signalling events relying on GTP-binding protein activation. For instance, the exocytotic mechanism of permeabilized rat mast cells (Pinxteren et al., 2000; Martin et al., 1995; Lillie and Gomperts, 1992) and rat PC12 (pheochromocytoma cells) cells (Vu and Wagner, 1993) was shown to be dependent on low concentrations of GDP (< 100 μM) and was inhibited with the addition of higher concentrations of GDP. The positive effect of GDP in the lower concentration range was suggested to be due to its conversion to GTP by the NDPK. The involvement of the NDPK in secretion was supported by the requirement for ATP in the permeabilized cells (Martin et al., 1995;
Vu and Wagner, 1993) and also by the finding that the addition of NDPK to ATP-depleted cells partially restored ATP[S]-stimulated secretion (Vu and Wagner, 1993).

Overall, the data presented in this chapter clearly lay emphasis on an exclusive reconstitution of a ternary complex involving GEFs, ARFs and PLD in both standard permeabilized HL-60 cells and in *in vitro* model systems. The apparent discrepancy between these two systems revealed by the failure of the same GEFs to substantially elevate the ARF-stimulated PLD *in vitro* strongly indicate the difficulty in mimicking the natural environment of the cell in *in vitro* assays. This is further complicated by the multifunctional nature of some the regulatory molecules such as polyphosphoinositides involved in PLD activation. Importantly, one of the highlights of the work presented in this chapter is the fact that the entire control sequence from PIPₙ → ARF-GEF → ARF → PLD1 can be reproduced in permeabilized HL-60 cells. Moreover, traditional views on the positive stimulatory effect of bulk, diffuse guanine nucleotide are also challenged by the finding that GTP could exert inhibitory effects under specific conditions. In addition, it emerged that GDP seems to be preferentially used as a source of GTP by ARFs and GEFs to effectively activate PLD in the permeabilized cells. The molecular basis for this observation will need further investigation.
Chapter six

General discussion
The principal theme of this thesis is the coordinated regulation of PLD by ARFs and its small exchange factors. This has been examined by using two different but complementary model experimental systems in order to allow direct comparisons. The requirement for such an investigation into the regulation of PLD, at the molecular level, stems principally from the fact that there is still not a clear definition of the discrete steps coupling receptor stimulation at the cell surface to the activation of PLD. In addition, contentions over the specificity and selectivity of the necessary protein-protein and protein-lipid interactions still exist in the published literature and are, on the whole, the norm. This thesis thoroughly addressed some of the fundamental issues and generated new insights that will be useful for the understanding of this complex type of coordinated regulation common in cell physiology. This brief chapter will summarize the new findings, including their originality, and also further consider the value and the limitations of the permeabilization/reconstitution system as a tool for the study of the cell-signalling events that regulate PLD.

The data presented in this thesis indeed confirmed the well-established view that myristoylation at the N-terminus of ARFs plays a crucial role in ARF functions i.e. recruitment on cellular membranes, guanine nucleotide binding property, interaction with effector and regulator proteins. Furthermore, this was done side-by-side, with identical materials, allowing the inferences drawn from one piece of work to be extended confidently to be into others. I have proposed a largely collision-based mechanism as a feasible explanation for the intriguing guanine-nucleotide-independent ARF stimulation of PLD activity in the permeabilised-cell model system. Alternatively, a pool of endogenous GTP may be responsible for the activation of
ARF exogenous. The loading of endogenous GTP on ARF may be catalyzed by residual endogenous ARF-GEFs. In addition, it is possible that endogenous NDPK converts the endogenous pool of GDP into GTP leading to the activation of recombinant ARF in the reconstituted cell-based system. Overall, the use of a fully-myristoylated recombinant ARF protein in permeabilized cells makes it possible to reconstitute PLD activation without the need to add exogenous GTP.

The determination of a full extent of myristoylation the ARFs used made it possible to show a clear-cut difference between ARF1 and ARF6 in PLD regulation. Indeed, ARF1 turned out to be unequivocally a better activator of PLD than ARF6 and this feature was observed in the two model systems used in this thesis. As a result, it is probable that these two proteins perform different non-redundant functions in vivo.

The data reported in this thesis clearly demonstrated that the small ARF-GEFs enhanced guanine nucleotide exchange on ARF1 and ARF6, preferentially on ARF1. However, the same small ARF-GEFs did not preferentially interact with PIP3 over PIP2 in vitro. My rationalisation for this apparent contradiction of the currently accepted view of an ARF-GEF selectivity towards PIP3 is based on the different experimental conditions and different model systems used in each case. Notably, the fact that the small ARF-GEFs cannot discriminate between PIP3 and PIP2 under the experimental conditions described in this thesis cannot be readily attributed to the small differences in the PH domains. This would seem to imply that the small ARF-GEFS are functionally redundant in terms of recognizing changes in the levels of these two specific phosphoinositide and transmitting this signal to PLD. Alternatively, the participation of other partner proteins, possibly interacting with the coiled-coil domain
of the ARF-GEFs, might lend specificity and selectivity towards phosphoinositides. Conceivably, this would enhance protein targeting to the cell membranes and subsequent activation of ARFs. In my work this hypothesis is confirmed by the observation that PIP₃ supports PLD activation better than PIP₂ in permeabilised HL-60 cells. So, in accord with other models, the fact that either PIP₃ or PIP₂ are strongly involved in the regulation of the interaction of the small ARF-GEFs with phospholipid membranes indicates the possible participation of upstream signalling pathways responsible for the synthesis of these polyphosphoinositides. Ultimately, and conventionally, these signalling events may synergistically control the activation of PLD in response to cell stimulation by extracellular factors.

Much of the originality of the work described here lies in the novel reconstitution of a ternary signaling system consisting of GEFs, ARFs, and PLD in both cell-free and permeabilized cell assays. As far as I am aware, there have been no previous reports on the reconstitution of such a ternary complex. The same is true of the quaternary system that was established by mixing polyphosphoinositides, GEFs, ARFs and PLD in these model systems. Specifically, it was discovered that the small ARF-GEFs strongly enhanced PLD activation mediated by ARF1. By contrast, these small ARF-GEFs had no effects on PLD activity in vitro. Interestingly, polyphosphoinositides in both systems substantiated the ARF-regulated PLD activity and therefore implicating other pathways in the regulation of PLD in vivo.

Thus the observation of a coordinated regulation of PLD that is not obvious in vitro but is clearly revealed in a cellular context, i.e. permeabilized cells, lays emphasis on the important concept of emergent properties in biological systems. This
concept is illustrated in my work by the finding that PIP$_3$, for which there is apparently no specificity expressed \textit{in vitro} by the small ARF-GEFs, turned out to be a better activator of PLD than PIP$_2$. It seems likely that in a larger, more complicated system than that available in cell-free assays, PIP$_3$ does not act exclusively on any one individual step upstream of PLD e.g. the ARF-GEFs, but probably modulates the combined, simultaneous effects of several PLD regulators. Thus the selectivity of the system emerges and clearly shows a preference for PIP$_3$ over PIP$_2$. The emergent selectivity for PIP$_3$ over PIP$_2$ for PLD regulation is indeed puzzling since the level of PIP$_2$ is presumed to be considerably higher in cells, even if the concentration of PIP$_3$ is transiently elevated in response to cell stimulation. It is probable that processes that stimulate PI 3-kinase also trigger a decrease in the level of PIP$_2$ by sequestering it into specialized cellular membranes compartments such as caveolae and membrane ruffles or causing its hydrolysis by PLC. Similarly, the results showing that at high protein concentrations ARF1 is a stronger PLD activator than ARF6 is of special interest. This is because ARF1 is known to accumulate to extremely high concentrations in Golgi membranes, for example, where it is present in transport vesicle coats in stoicheometric ratios of 3:1 to coatomer proteins.

The other original feature of this work is the use of two independent experimental systems namely the cell-free and cell-based systems to carry out the study of PLD regulation. As shown in this thesis, the cell-free system allows the characterization of molecular interactions but unfortunately lacks many details of intracellular structure and regulation. I contend that this explains the absence of the stimulatory effects of the small-GEFs on PLD activity \textit{in vitro}. By contrast, the second
system is a cell-based system and therefore more physiological. It essentially depends on the dual permeabilization/reconstitution process which has proven to be a valuable tool for the biochemical dissection of signalling pathways that regulate PLD activity within neutrophil-related cell lines. Indeed, the gentle permeabilization step by the bacterial cytolysin, SLO, generates pores in the plasma membrane of the cell, allowing the rapid efflux of cytosolic proteins while maintaining of the general structural features. In such a system, I have confidently demonstrated, for instance, that the use of exogenous GDP as a source for GTP is a more sensitive way to activate ARF proteins than the use of either GTP or GTPγS. As a result, this novel observation should prove useful for any future investigation into the regulation of PLD by small GTP-binding proteins in a permeabilization/reconstitution system. Finally, it is reasonable to think that the permeabilized HL-60 cell system may be adapted to other specialized cell types in order to help further clarify the complex mechanisms of regulation of PLD.

However, the use of a permeabilization/reconstitution system has its own limitations. The permeabilization procedure allows the leakage of a large proportion of cytosolic components that may be crucial for the control and regulation of the signalling pathway under investigation. In addition, cross-talk mechanisms probably operate under certain conditions which are difficult to assess in a cell permeabilization assay. The other disadvantage of SLO permeabilization is the size restriction of molecules that can pass through the lesions (molecules > 150 kDa cannot exit easily from the permeabilized cells). Thus the major concerns in using this cell-based reconstituted system remains that it has always been difficult to assess to which extent
the permeabilized cells are a valid model for the intact cell. As already mentioned, cell permeabilization is an excellent step towards dissecting molecular processes but there is always a reasonable concern that important cellular structures, such as the cytoskeleton, or membrane organelles can be affected during permeabilization and therefore permeabilized cells are by no means perfect models for the living wild-type cells. However, this type of reasonable concern about a perturbation to an experimental system must always be a part of modern cell biology. For example, the use of transformed cell lines, the microinjection of recombinant proteins or of RNA or DNA, the transfection and overexpression of foreign DNA under strong promoters, all those techniques bring specific artifacts to any investigation. Hence, biological problems are best solved by bringing several complementary techniques to bear simultaneously.

Figure 6.1 schematically presents the different aspects of the regulation of PLD at the molecular level described in this thesis.

Continuation of the work described here might focus on the following issues.

First, it would be interesting to characterize the discrepancy between ARF1 and ARF6 with respect to their interaction with polyphosphoinositides in order to determine whether the observed specificity of ARF1 towards PLD could be due to specific interactions with polyphosphoinositides. This protein-lipid interaction could be investigated by using the liposome-binding assay in which lipid vesicles supplemented with the polyphosphoinositides of interest would be exposed to the ARF proteins. In addition, a qualitative overlay assay using polyphosphoinositides bound to nitrocellulose membranes could be carried out.
Figure 6.1. Overview of the different signalling pathways leading to PLD activation in cells.
Second, localisation studies could be performed in permeabilized HL-60 cells to examine whether ARF1 and ARF6 are in fact differently localized in these cells. This would require the purification and expression of epitope-tagged ARF proteins. Because ARF proteins must be myristoylated to retain biological activity, epitope tagging must be restricted to the carboxyl end of the molecule. Small tags such as haemaglutinin (HA) and larger ones like green fluorescent protein (GFP) could be added to the carboxyl terminus. Detection and visualization could be performed by specific antibodies and fluorescence microscopy.

Third, it is crucial to elucidate the specific functional role(s) of the different GDP and GTP-bound states of ARF1 in the regulation of PLD activity. One of the hallmarks of this project was the observation that ARF1 at high concentrations could activate PLD without the requirement for exogenous GTP. As a consequence, an important question arises as to whether a fully-myristoylated form of ARF1 (T31N), a mutant that preferentially binds GDP, could activate PLD in the permeabilized cell system. This experiment would reveal whether the GTP-dependent reorganization of the ARF switch regions is required for PLD activation. An answer to this question will undoubtedly bring more insight into the functions of ARF in controlling PLD activation.


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