REVIEW ARTICLE

Phospholipase D: molecular and cell biology of a novel gene family

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Interaction of extracellular-signal molecules with cell-surface receptors often activates a phospholipase D (PLD)-mediated hydrolysis of phosphatidylcholine and other phospholipids, generating phosphatidic acid. The activation of PLD is believed to play an important role in the regulation of cell function and cell fate. Multiple PLD activities were characterized in eukaryotic cells, and, more recently, several PLD genes have been cloned. A PLD gene superfamily, defined by a number of structural domains and sequence motifs, also includes phosphatidyltransferases and certain phosphodiesterases. Among the eukaryotic PLD genes are those from mammals, nematodes, fungi and plants. The present review focuses on the structure, localization, regulation and possible functions of cloned mammalian and yeast PLDs. In addition, an overview of plant PLD genes, and of several distinct PLD activities that have not yet been cloned, is provided. Emerging evidence from recent work employing new molecular tools indicates that different PLD isoforms are localized in distinct cellular organelles, where they are likely to serve diverse functions in signal transduction, membrane vesicle trafficking and cytoskeletal dynamics.

Key words: membrane traffic, phosphatidic acid, phospholipase D, phospholipid metabolism, signal transduction.

SIGNAL-DEPENDENT ACTIVATION OF PHOSPHOLIPASE D

Phospholipase D (EC 3.1.4.4; PLD) was first discovered in plants as a distinct phospholipid-specific phosphodiesterase activity that hydrolyses phosphatidylcholine (PC) to phosphatidic acid (PA) and choline (Scheme 1; [1,2]). However, widespread interest in this enzyme had only begun once experiments in cultured animal cells revealed its rapid and dramatic activation in response to extracellular stimuli [3–7]. These and other early studies were reviewed by Exton [8] and Billah [9] and have indicated that PLD is a *bona fide* 'signal-activated' phospholipase. As such, PLD was conceptually grouped together with phosphoinositide-specific phospholipase C, phospholipase A_2 and sphingomyelinase; all are phospholipid-degrading enzymes that generate biologically active products which are assumed to play important functions in cell regulation [10]. Signal-activated phospholipases and lipid kinases together represent the molecular basis for the rapidly growing field of lipid signalling (reviewed in [11–14]). The activity of these enzymes is stimulated upon receptor ligation by agonists, resulting in modification of various lipid constituents of the membrane, either by degradation or by phosphorylation, and generation of one or more products ('messengers') that are able to recruit or modulate specific target proteins.

In common with many esterases, besides simple hydrolysis PLD may catalyse a transesterification reaction (in this case of transphosphatidylation), utilizing short-chain primary alcohols as phosphatidyl-group acceptors [15–17]. The resultant phosphatidylalcohols are produced only by PLDs, and are not normally found in biological membranes. Because of their unique origin, their low basal levels and their relative metabolic stability, the formation of phosphatidylalcohols has served as a convenient and sensitive marker for PLD activation in cultured cells (see [18] for a methodological review). Furthermore, alcohols have been employed to probe the involvement of PLD in various regulatory processes, because of their ability to decrease PA formation by shunting phosphatidyl moieties into phosphatidylalcohols (Scheme 1). The signal-dependent activation of PLD was demonstrated in numerous cell types stimulated with various hormones, growth factors, cytokines, neurotransmitters, adhesion molecules, drugs and physical stimuli (Table 1 provides some recent examples). Studies into the mechanisms of receptor–PLD coupling have implicated multiple pathways leading to PLD activation, that include protein serine/threonine kinases (e.g. protein kinase C), small GTPases (e.g. ADP-ribosylation factor, RhoA and Ral) and tyrosine kinases [19–22]. It is generally assumed that PA is the major messenger molecule generated by the PC-specific enzymes [23,24]. (However, other PLD forms may produce water-soluble messenger molecules.) Whereas in many instances PA can modulate enzyme activity *in vitro* [23], its molecular targets *in situ* have yet to be identified with any degree of certainty.

In recent years, research on PLD has turned increasingly away from phenomenology and regulation and has moved into the molecular cell biology of the structure and function of PLD isoenzymes in eukaryotic cells. In addition to reviews on specific aspects of PLD research that are cited above, a number of excellent, general reviews were recently published [25–29]. The

Abbreviations used: ARF, ADP-ribosylation factor; BFA, brefeldin A; DAG, diacylglycerol; EGF-R, epidermal-growth-factor receptor; ER, endoplasmic reticulum; EST, expressed sequence tag; fMet-Leu-Phe, formylmethionyl-leucylphenylalanine; GFP, green fluorescent protein; GPI, glycosylphosphatidylinositol; GTP[S], guanosine 5′-[γ-thio]triphosphate; MAP kinase, mitogen-activated protein kinase; MMP, matrix metalloprotease; ORF, open reading frame; PA, phosphatidic acid; PC, phosphatidylcholine; PDGF, platelet-derived growth factor; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLD, phospholipase D; PS, phosphatidylserine; TGN, *trans*-Golgi network.

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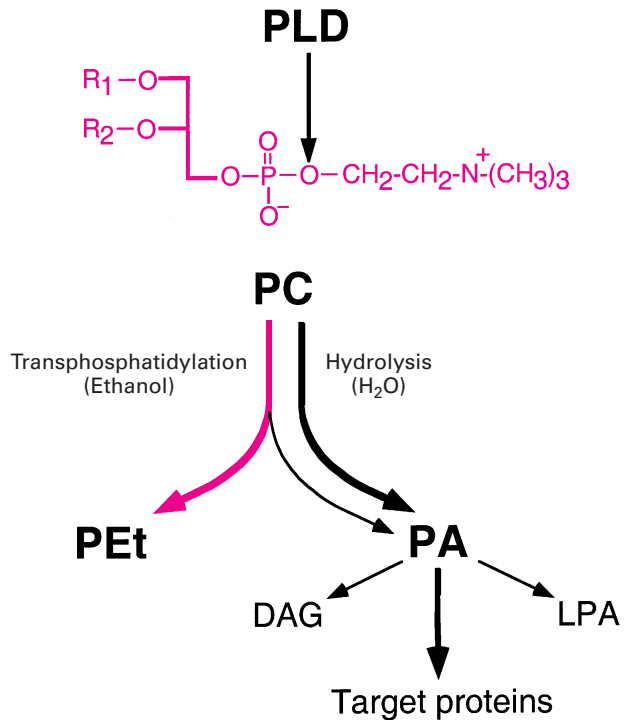

Scheme 1 Phospholipase D-catalysed reactions

PLD hydrolyses the distal phosphodiester bond in phospholipids such as PC (the structure shown above in red). A phosphatidyl-enzyme intermediate is believed to form transiently which normally is hydrolysed by water, generating PA. Primary short-chain alcohols (e.g. ethanol) can substitute for water in a competing, transphosphatidylation, reaction. In the presence of ethanol the product of PLD-catalysed transphosphatidylation is PA ethyl ester or phosphatidylethanol (PEt). This reaction (thick red arrow pointing to the left) occurs at the expense of the hydrolytic reaction (thin black arrow pointing to the right), decreasing PA formation. PA can also be produced by diacylglycerol kinase and by acylation of glycerol 3-phosphate. In contrast, phosphatidylalcohols are uniquely formed by PLD. PA can be further metabolized (thin black arrows) to diacylglycerol (DAG) and lyso-PA (LPA). In contrast, phosphatidylalcohols are metabolically stable and would accumulate in cells upon PLD activation. Because cellular phosphatidylalcohol levels are normally extremely low, their accumulation upon PLD activation is readily detectable. These properties have made phosphatidylalcohols useful markers of PLD activation *in vitro* and *in vivo*. Attenuation of PLD-catalysed formation of PA by trapping the phosphatidyl moiety in a biologically inactive phosphatidylalcohol has enabled 'alcohol trap' experiments designed to establish the role of PLD in various physiological responses. It should be noted that although phosphatidylalcohols are formed only by PLDs, not all PLDs can catalyse this reaction [148,149,183,237].

present review will focus on the structure, regulation, localization and proposed function(s) of cloned yeast and mammalian PLD gene products that have been studied extensively during the last 5 years.

THE PHOSPHOLIPASE D GENE FAMILY

Cloning the first PC-PLD genes

Plant PLD has continued to play an important role in the unfolding story of PLD research, as it became the first eukaryotic PC-hydrolysing PLD to be purified and subsequently cloned, opening the way for yeast and mammalian PLD genes that, together, defined the PLD gene family. Generally, eukaryotic PLDs proved to be sensitive enzymes that were hard to purify. Mammalian PLDs, in particular, have resisted many attempts at purification. Only one enzyme, the oleate-dependent PLD from porcine lung, reportedly was purified to homogeneity [30]. In contrast, a plant PLD was successfully purified in two laboratories, independently, from cabbage (*Brassica oleracea*) and castor bean (*Ricinus communis*) [31,32]. N-terminal sequence analysis of the two proteins revealed that they shared a nearly identical 10-amino-acid N-terminal peptide sequence ($^{I}/_{V}EET^{V}/_{I}GFGKG$). On the basis of that sequence, Xuemin Wang and his colleagues have isolated a castor-bean endosperm cDNA that encoded the first eukaryotic PC-specific PLD to be cloned. This cDNA encoded a 92 kDa protein that exhibited both hydrolytic and transphosphatidylating Ca^{2+}-dependent PLD activity with PC as substrate [33]. Significantly, the plant PC-PLD (later termed PLD-α) had no sequence similarity to a previously cloned, PLD-type serum enzyme that exclusively cleaves glycosylphosphatidylinositol (GPI)-anchored proteins [34]. In fact, with the exception of an N-terminal domain, similar to the C2 domains of protein kinase C and other proteins, plant PLD-α was similar to no known protein.

The cloning of plant PLD-α was a breakthrough in that it provided a prototype sequence that enabled identification of other eukaryotic PLD sequences based on sequence similarity. A search of the GenBank® database, utilizing plant PLD-α as a query sequence, immediately revealed a homologous yeast hypothetical open reading frame (ORF) (YKR031c), obtained by systematic sequencing of chromosome 11 as part of the yeast genome project. YKR031c was readily identified as a gene that encodes a Ca^{2+}-independent PLD activity and was provisionally termed yeast PLD1 [35,36]. In parallel, Engebrecht and colleagues have cloned and sequenced a yeast sporulation gene called *SPO14*, that turned out to be similar to that coding for plant PLD-α and identical with YKR031c, and that also was identified as a PLD-encoding gene by genetic disruption and heterologous expression [37]. On the basis of the plant and yeast genes, a human PLD cDNA was cloned and termed PLD1 [38]. Together, the plant, yeast and human PLD genes comprised a new gene family; the limited but significant similarity shared among them defined a number of characteristic, highly conserved sequence motifs [38,39]. These PLD genes all belong to an extended gene superfamily that also includes bacterial PLDs, phosphatidyltransferases/phospholipid synthases, endonucleases and certain viral envelope proteins and their mammalian counterparts [39–42]. PLD superfamily members all share a conserved motif ($HXKX_4DX_6G^{G}/_{S}$) that is involved in catalysis and that confers a similar mechanism of action (see below).

Phylogenetic relationship and domain structure

Table 2 provides a summary of eukaryotic PLD genes that have been cloned or have been identified as full-length ORFs in genome projects. Among these genes are two mammalian PLDs that have been termed PLD1 [38,43] and PLD2 [44,45]. Fungal PLD genes include one from *Saccharomyces cerevisiae* (baker's yeast), namely *SPO14/PLD1* [35–37] and a highly related PLD from *Candida albicans* [46]. Plant PLDs that have so far been cloned represent three forms, namely PLDα, PLDβ and PLDγ [47,48]. In addition, protein sequence databases contain the complete coding sequence of several putative but highly probable PLD genes, including that of the fission yeast *Schizosaccharomyces pombe* (accession Q09706), which is very similar to *SPO14/PLD1*, and a mammalian-like PLD from the nematode *Caenorhabditis elegans* (accession U55854). Three bacterial PLDs, from *Streptomyces antibioticus*, *Streptoverticillium cinnamoneum* and *Yersinia pestis*, are also included in Table 2. A progressive global alignment of these sequences was carried out using the CLUSTAL W software [49]. The resultant multiple sequence alignment was validated by comparison with an align-

Table 1 Signal-dependent activation of PLD: some recent examples

Stimulus	Cell type	Reference
Neurotransmitters		
Cholinergic agonists	Rat pheochromocytoma PC12 cells	[215]
Glutamatergic agonists	Rat hippocampal slices	[216]
Histamine	Human 1321N1 astrocytoma cells	[81]
Bradykinin	Human 1321N1 astrocytoma cells	[81]
Noradrenaline	Primary rat cortical astrocytes	[82]
Hormones		
Vasopressin	Rat vascular smooth-muscle A7r5 cells	[217]
Gonadotropin-releasing hormone	Murine αT3-1 gonadotrophs	[218]
Extracellular matrix		
Collagen, laminin and fibronectin	Murine mammary adenocarcinoma LM3 cells	[219]
Thrombin	Human platelets	[220]
Bioactive lipids		
Lyso-PA	PC-3 human prostate-cancer cells	[221]
Platelet-activating factor	Human eosinophils	[208]
Growth factors		
PDGF	HIRcB fibroblasts	[84]
EGF	Rat calvarial osteoblastic cells	[222]
Chemoattractants		
fMet-Leu-Phe	Human neutrophils	[223]
Eicosanoids		
Leukotriene B_4	Human neutrophils	[224]
Thromboxane A_2	Human platelets	[220]
Cytokines/chemokines		
Tumour necrosis factor-α	Murine B cell lymphoma A20 cells	[225]
Fas ligand	Human premyelocyte HL-60	[225]
RANTES	Human Jurkat T cells	[83]
Reactive oxygen species		
H_2O_2 lipid ozonation products	Rat pheochromocytoma PC12 cells	[215]
Bacterial toxins		
Lipopolysaccharide	Human macrophages	[226]
Cell-surface receptors		
Antigen	Guinea-pig lung mast cells	[227]
IgG receptor (Fc-Rs)	Human macrophages	[228]
Other stimuli		
Apolipoprotein A-I	Human fibroblasts	[229]
High glucose	Rabbit coronary vascular smooth-muscle cells	[230]
Monosodium urate	Human neutrophils	[231]
Mechanical stretch	Rat skeletal muscle	[232]
γ-Radiation	Human squamous-carcinoma SQ-20B cells	[233]

ment of representative animal, plant and yeast PLDs based on numerous pairwise BLAST analyses of the individual sequences. The multiple sequence alignment thus obtained was utilized to generate an unrooted phylogenetic tree (Figure 1). The PLD family tree consists of three major limbs: (i) the bacterial PLDs (among which Ymt, the PLD from *Yersinia pestis*, is very divergent); (ii) plant PLDs; (iii) fungal and animal PLDs. Within the last cluster, fungal PLDs are clearly divergent from the animal (i.e. mammalian and nematode) PLDs.

Alignment of five PLD sequences from representative vertebrate, invertebrate, fungal, plant and bacterial species reveal some notable features of this gene family (Figure 2). All eukaryotic PC-PLD genes cloned to date share a relatively conserved catalytic core flanked with much less conserved N- and C-terminal regions. The catalytic core of all eukaryotic PLDs is comprised of domains I–IV (shown in red in the Figure; [39]). These domains are found also in the bacterial PLD. There is significant internal similarity, in four short sequence motifs, between domains I plus II and domains III plus IV [41]. This suggests that eukaryotic PLD genes evolved from an ancestral gene that underwent a gene duplication and fusion event, raising the possibility that PLDs are bilobed enzymes [41]. The presence of non-conserved inserts of different lengths between conserved domains II and III (Figure 2) supports this suggestion. This extended insert or 'loop' region was shown to be dispensable for activity in human PLD1 [50]. Furthermore, co-expression of the individually inactive, N-terminal half of rat PLD1 (comprising domains I and II) with the C-terminal half (comprising domains II, III and IV), restored full PLD activity *in vivo* [51]. This indicates that PLD activity requires conserved motif(s) that are present in the two halves of the molecule and that these two regions can associate correctly to reconstitute the catalytic centre.

In addition to domains I–IV, additional domains (Figure 2, blue) are conserved in the yeast, human and nematode sequences, but are absent from the plant and the bacterial PLDs. The human and nematode PLDs both contain a region in their N-terminal half that has been identified as a PX (*phox*) domain (Figure 2, green; [52]). In the nematode PLD, this PX domain includes a long insert that is not found in other PX domains. The function of the PX domain is unknown; it is not required for catalytic activity [50]. Plant PLDs exhibit an N-terminal C2 domain (also known as a Ca^{2+}-lipid binding domain; Figure 2, yellow). C2 domains are found in a wide range of proteins. Some prominent examples are cytosolic phospholipase A_2, protein kinase C isoenzymes, a phosphatidylinositol 3-kinase isoenzyme and synaptotagmin (see [53]). C2 domains are believed to confer upon these proteins the ability to bind acidic phospholipids in a Ca^{2+}-dependent manner [54]. Indeed, plant PLDs require micromolar Ca^{2+} for activity, a requirement which may well be mediated by their C2 domain. However, C2 domains may be involved also in binding to specific acceptor proteins and thus participate in recruitment of C2-containing proteins to specific subcellular compartments [55,56].

Catalysis in PLD superfamily members

Domain IV contains a relatively short sequence motif, $HXKX_4DX_5IGSXN$, termed the 'HKD motif' [38] or the phosphatidyltransferase motif [35]. A variant form of the HKD/phosphatidyltransferase motif appears also in domain II of PLDs. The HKD motif is particularly well conserved in eukaryotic and bacterial PC-PLDs, but it can be found also in other PLD superfamily members such as non-PLD phosphatidyltransferases (e.g. cardiolipin synthase and phosphatidylserine synthase), as well as in certain pox-virus envelope proteins and several endonucleases [39,41]. Because of their high degree of conservation, it has been predicted that the histidine, lysine, aspartate, glycine, and serine residues in HKD motifs play an important role in catalysis. Mutagenesis studies in human PLD1 have largely confirmed this prediction [57]. The catalytic mechanism of PLD has long been suspected to involve a phosphatidyl-enzyme intermediate [15]. Recent studies, in an endonuclease PLD superfamily member and in the *Yersinia pestis* murine toxin (which has PLD activity), have identified the histidine in the HKD motif as a nucleophile that forms a covalent phosphohistidine intermediate [58,59]. These results suggest that, in PLDs, this histidine residue attaches the phosphatidyl moiety to form a transient phosphatidyl-enzyme intermediate, which is then subject to attack by an activated water molecule (or a primary short-chain alcohol) to release PA (or a phosphatidyl-alcohol) [59]. The crystal structure of Nuc, a bacterial endo-

Table 2 Representative eukaryotic and prokaryotic PLD genes

Name	Description	Species	Accession no.	Reference
PLD1	ARF/Rho/PKC-dependent PC-PLD	*H. sapiens*	AAB49031	[38]
		*R. norvegicus**	U69550	[43]
PLD2	PIP_2/ARF-dependent PC-PLD	*M. musculus*†	U87557	[44]
		R. norvegicus	BAA19882	[45]
CePLD (ORF)	Putative PLD	*C. elegans*	AAA98011	GenBank®
SPO14/PLD1	Ca^{2+}-independent, PIP_2-dependent PLD	*S. cerevisiae*	S38103	[37]
CaPLD	Ca^{2+}-independent PLD	*C. albicans*	BAA32278	[46]
YA2G (ORF)	Putative PLD	*S. pombe*	Q09706	GenBank®
PLDα	Ca^{2+}-dependent PC-PLD	*R. communis*	Q41142	[33]
		A. thaliana	AAC49274	[234]
PLDβ	Phosphoinositide-dependent PLD	*A. thaliana*	AAC49656	[234]
PLDγ	Phosphoinositide-dependent PLD	*A. thaliana*	AAB87672	[48]
SaPLD	Bacterial PLDs	*S. antibioticus*	BAA03913	[235]
SciPLD		*S. cinnamoneum*	BAA75216	[236]
Ymt		*Y. pestis*	AAC82729	[59]

* *Rattus norvegicus.*
† *Mus musculus.*

nuclease that belongs to the PLD gene superfamily, was reported recently [60]. Nuc monomers comprise a β-sheet made of eight β-strands that is flanked by five α-helices. Nuc crystallizes as a dimer to form a saddle-like structure that is hypothesized to straddle the phosphate backbone of DNA in a way that enables contact between the phosphodiester bond and the active-site residues of the HKD motif [60]. The position of the active site's conserved histidine residue is consistent with its role as the nucleophile that forms a phosphoenzyme intermediate during catalysis [60].

In conclusion, eukaryotic PC-specific PLD genes identified to date represent several distinct but related molecular forms. It may be expected that additional isoforms will be isolated in coming years. It remains to be seen if, and to what extent, novel mammalian PLD genes would be modelled after the non-mammalian prototypes depicted in Figure 2. The extent to which different isoforms are co-expressed in individual cell types is not yet known. On the basis of current knowledge of other multigene families, it may be predicted that individual cell types would express several (but not all) PLD isoforms, each with its own mode of regulation, subcellular localization and function. This prediction is beginning to be confirmed by recent data on the mammalian PLD1 and PLD2 isoenzymes.

MAMMALIAN PLD1

Structural features of PLD1

The mammalian PLD1 cDNA was cloned from a HeLa cell cDNA library and found to encode a 1074-amino-acid protein [38]. The expressed PLD1 exhibits PC-specific PLD activity (hydrolysis and transphosphatidylation) and is activated by ADP-ribosylation factor (ARF) and by phosphatidylinositol 4,5-bisphosphate (PIP_2) [38]. Hence, PLD1 is generally believed to represent the major ARF-dependent PLD activity that has been studied extensively since the discovery of ARF as a small G-protein that activates PLD in membranes and cell ghosts [61,62]. PLD1 has been renamed PLD1a after another splice variant has been identified, PLD1b, that lacks a 38-amino-acid region [43,63–65]. The domain structure of PLD1 is generally similar to that of PLD2 and CePLD (Figure 2). It contains the four core domains I–IV, a putative PX domain, and a C-terminal motif which is conserved in all eukaryotic PLDs. Unlike PLD2 (but similar to the nematode putative PLD), PLD1 has a 116-amino-acid 'loop' between domains I and II. As expected, mutations within both HKD motifs abolish PLD activity [57]. Deletion of an N-terminal region comprising 325 amino acids results in a stable protein that exhibits nearly intact basal activity and slightly enhanced ARF- and Rac1-dependent activity, indicating that this N-terminal fragment is dispensable for PLD1 catalytic activity [50]. Similarly, deletion of the central loop region (Δ505–620) has no negative effect on PLD1 activity. In contrast, deletion of the C-terminal 99 amino acids (Δ976–1074) abolishes all PLD activity [50]. The loop region of PLD1 is highly variable even among different mammalian PLD1 sequences, and thus is likely to act as a 'spacer' that allows requisite conformational changes upon binding of activator proteins [50].

A putative PX domain that is located in the N-terminal region of the molecule (residues 99–213) was recognized in PLD1 [52]. Because a truncated PLD1 (PLD1-Δ1–325) is enzymically active [50], it may be assumed that the PX domain plays a regulatory role in either intra- or inter-molecular interactions. PLD1 is post-translationally modified. Rat PLD1b is a glycoprotein, the N-glycosylation of which appears to determine its targeting to a membrane compartment [66]. Human PLD1a is palmitoylated, and this fatty acylation appears to be essential for enzymic activity [67].

Tissue distribution and regulation of PLD1 expression

PLD1 is highly expressed in kidney and lung, but detectable levels were also observed in other tissues [68]. In rat, PLD1b is the major expressed form and is found at high levels in the kidney, small intestine, colon and liver; PLD1a is mainly expressed in the lung, heart and spleen [65]. Very low levels of either form were seen in the testis, thymus and muscle. In accord with the differential expression of PLD1 in tissues, the expression level of PLD1 appears to be under stringent developmental control. Induction of C6-glioma-cell differentiation results in a transient elevation of PLD1a expression and, concurrently, a decrease in expression of PLD1b [63]. PLD1a and PLD1b mRNA levels were elevated in nerve-growth-factor-treated PC-12 cells [69]. Similarly, granulocytic differentiation of HL-60 cells induces elevated expression of both PLD1a and PLD1b [70]. Differentiation of epidermal keratinocytes in response to 1,25-

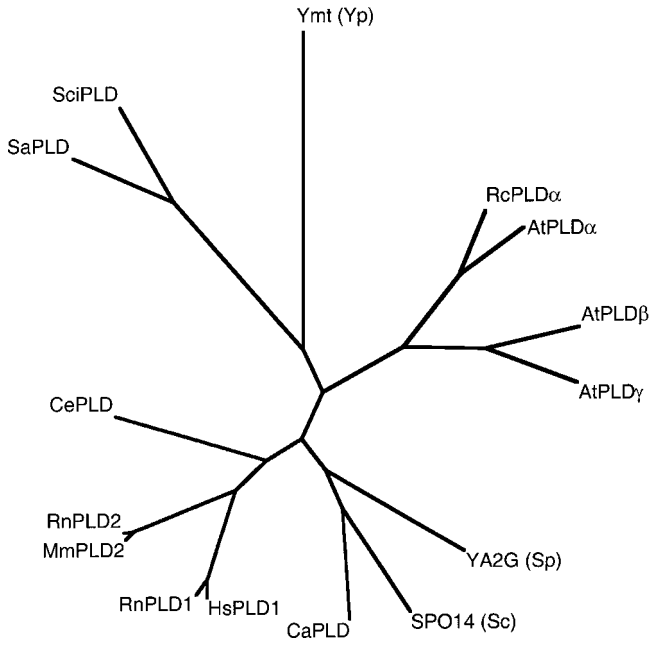

Figure 1 Phylogenetic tree of the PLD family

A progressive global alignment of the 15 eukaryotic and prokaryotic PLD sequences listed in Table 2 was carried out using the CLUSTAL W software, with the gaps excluded option On [49]. The multiple sequence alignment thus obtained was utilized to generate an unrooted phylogenetic tree using the Drawtree routine of the Phylip package [238].

dihydroxyvitamin D_3 is also associated with increased expression of PLD1a [71]. In rat brain, PLD1a expression is elevated *in vivo* during days 5–15 of postnatal development, a time that coincides with the major period of synaptogenesis and myelination [72]. Together, these data support a role for PLD1 isoforms in regulation of differentiated cell functions in diverse cell types.

Regulation of PLD1 activity

The regulation of PLD activity by cytosolic factors has been amply reviewed [21,22]. Studies employing cell membranes and permeabilized cells have identified most of the cytosolic factors that affect PLD activity. These factors include several small GTPases such as ARF1 and its isoforms, RhoA, other Rho family members, and RalA. In addition, protein kinase C-α (PKC-α) was shown to regulate PLD activity in a phosphorylation-independent manner, through its regulatory domain. An unidentified factor of 50 kDa [73] and a 36 kDa protein, recently identified as the ganglioside G_{M2} activator protein [74], have also been implicated in regulation of PLD activity. Another important component in the PLD activation complex is PIP_2, a phospholipid that acts as a cofactor in PLD activation by the above molecules [61,75–78].

ARF and RhoA have been implicated in mediating agonist-induced activation of PLD *in vivo*. The evidence provided for ARF's involvement include the inhibition of PLD activation by brefeldin A (BFA), a fungal metabolite that blocks ARF-specific guanine nucleotide exchange proteins by locking them in an abortive complex with ARF·GDP [79]. BFA inhibits PLD activation by ligands of heptahelical G-protein-coupled receptors such as carbachol [80,81], bradykinin and histamine [81], noradrenaline [82] and RANTES [83]. In addition, BFA inhibits PLD activation by ligands of receptor tyrosine kinases such as insulin and platelet-derived growth factor (PDGF) [84,85]. An ARF-related protein that interacts with ARF guanine nucleotide exchange proteins attenuates muscarinic activation of PLD in HEK cells [86]. Finally, overexpression of arfaptin-1, a 39 kDa protein that associates with the GTP-bound form of ARF, attenuates the activation of PLD by phorbol ester [87]. Involvement of RhoA was suggested by inhibitory effects of bacterial toxins that modify and inactivate Rho proteins on activation of PLD [88,89]. RhoA-mediated activation of PLD involves Rho kinase, a Rho-dependent serine/threonine kinase [90], but its exact mode of action is unknown.

Numerous *in vitro* experiments with recombinantly expressed PLD1 gene products have led to the conclusion that PLD1 is the major PLD isoform that is regulated by ARF, PKC and RhoA [38,43,64–66,91]. However, it must be noted that PLD2 might also be regulated by ARF ([92,93]; see below). The site of interaction of PLD1 and ARF has not been positively identified, but is unlikely to be in the N-terminal or central loop regions of PLD1 [50]. In contrast, amino acids 1–325 are required for activation of PLD1 by PKC-α both *in vitro* [50] and *in vivo* [94]. Activation of PKC results in association of PLD1b with PKC-α and with another 220 kDa protein, and in serine/threonine phosphorylation of both p220 and PLD1 [95]. The identity of p220 is unknown; two possible candidates of similar molecular mass are BIG1, an ARF GDP/GTP exchange protein [96] and the 220 kDa splice variant of PI 4-kinase-α [97]. RhoA was shown to interact with a C-terminal fragment of PLD1 comprising amino acids 712–1074 by a yeast two-hybrid approach as well as by an *in vitro* co-precipitation assay [98]. None of the two RhoA-binding motifs identified in other effector proteins could be identified within the C-terminal fragment of PLD1, indicating that the PLD1–RhoA interaction motif is unique [98].

Another small GTPase that is involved in PLD1 activation is RalA. RalA binds to a PLD activity and may mediate in part activation of PLD in v-Src-transformed cells. Overexpression of RalA potentiates PLD activation by v-Src, and dominant negative RalA inhibits PLD activity in v-Src and v-Ras-transformed cells [99]. The PLD activity which can associate with RalA *in vitro* was identified as PLD1 [100]. PLD1 forms a complex with both ARF and RalA, and these GTPases directly interact with PLD1 at different sites [101]. Further work has shown the GTP-dependent formation of a RalA–ARF complex in lysates of Src- and Ras-transformed cells, that the complex contains PLD1, and that BFA inhibits PLD activation in Src- and Ras-transformed cells [102]. It is therefore probable that Ral GTPases are involved in PLD1 activation as anchor/adaptor proteins, but not as activators. Nevertheless, recent work does show that they play an essential role in phorbol-ester- and G-protein-mediated activation of PLD [103,104].

A puzzle that remains unsolved involves the site of interaction of PLD1 (and PLD2) with PIP_2. Steed et al. have identified a putative PH domain in the N-terminal region of both PLD1 ($hPLD1b_{220–329}$) and PLD2 ($hPLD2a_{203–312}$), albeit one that is radically different from classical PH domains of phospholipase C-δ1, spectrin, pleckstrin and dynamin [105]. The same domain was identified utilizing an advanced pairwise alignment algorithm (PSI-BLAST), and its significance was confirmed by comparison to the secondary and deduced three-dimensional structure of known PH domains [106]. However, it is still unclear whether this PH domain is necessary for PIP_2 interactions, because a truncated PLD, PLD1-Δ1–325, retains an absolute dependence on PIP_2 for activity [50]. The N-terminal PH domain may therefore mediate interactions with other entities, possibly with PKC (see [107] for review on PH domain interactions).

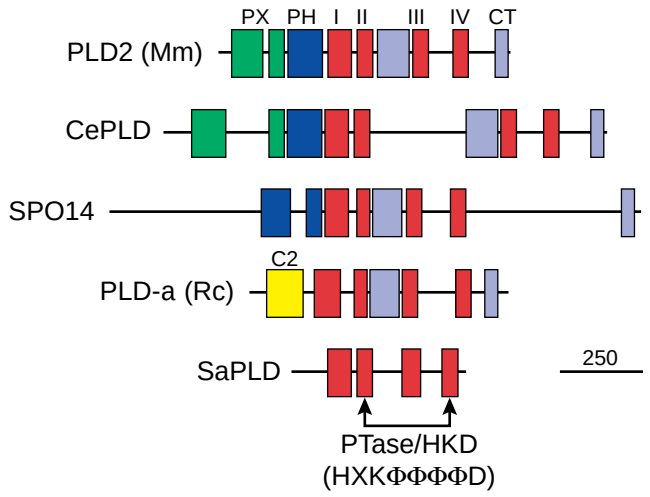

Figure 2 Domain structure of representative PLDs

Alignment of five PLD sequences from representative species of vertebrates [mouse (*Mus musculus*); PLD2], invertebrates (the nematode worm *Caenorhabditis elegans*; putative PLD), fungi [baker's yeast (*Saccharomyces cerevisiae*); SPO14/PLD1], plants [castor bean (*Ricinus communis*); PLD-α] and bacteria (*Streptomyces antibioticus*; SaPLD) reveal domains that are conserved among all PLDs as well as domains that are conserved only in eukaryotic PLDs. All PLDs have four conserved domains that have been designated domains I–IV [39,57] and are depicted as red boxes. A region between domains II and III and a short C-terminal domain (CT), both of which are conserved among all eukaryotic PLDs, are shown in light blue. A putative PX domain was identified in the N-terminal region of mammalian PLDs as well as the nematode putative PLD [52]. The PX (*phox*) domains are shown in green. The nematode sequence contains a large non-conserved insert within the PX domain. A putative PH domain that follows the PX domain and is found in mammalian, nematode and yeast sequences, but is absent from plant PLD-α, is depicted in dark blue [105,106]. Plant PLDs have an N-terminal C2 domain (also known as a Ca^{2+}-lipid binding domain (yellow box). C2 domains are found in a wide range of signalling proteins [53]) and are believed to be involved in binding acidic phospholipids in a Ca^{2+}-dependent manner [54] and to mediate recruitment of proteins to specific subcellular compartments [56]. There is significant internal homology in four sequence motifs between a region that corresponds to domains I plus II, and a region that corresponds to domains III plus IV, suggesting a gene duplication and fusion event [41]. Domains II and IV contain a short sequence motif ($HXKX_4DX_6G^G/_S$ termed HKD or the phosphatidyltransferase motif. The highly conserved histidine, lysine, aspartate, glycine and serine residues in the HKD/PT motif play an important role in catalysis [57,58].

Subcellular localization of PLD1

Subcellular fractionation and characterization studies have demonstrated the presence of ARF-dependent PLD activities in multiple cellular membranes, including the nuclear envelope, endoplasmic reticulum (ER), Golgi apparatus, transport/secretory vesicles and plasma membrane (see [108] for a review). Fluorescence-localization studies, which have utilized recombinant PLD1 tagged with green fluorescent protein (GFP) or Flu-epitopes, have provided isoform-specific information on PLD1 localization. In rat embryo fibroblasts, Flu-tagged PLD1a exhibits a perinuclear staining pattern, consistent with its localization in the ER, Golgi and late endosomes [44]. A similar localization was observed for GFP–PLD1a expressed in COS-7 cells [50]. Localization in intracellular membranes, possibly representing ER, Golgi and a vesicular compartment, also was reported for GFP–PLD1 in 3Y1 fibroblasts, although in this study GFP–PLD1 was found to localize also to the plasma membrane [109]. In all the above studies no attempt was made to verify the co-localization of PLD1 with organelle-specific marker proteins. It is therefore of interest that, in COS-1 and RBL-2H3 cells, recombinant GFP–PLD1b does not co-localize with a Golgi marker protein [TGN38 (*trans*-Golgi network 38)] but, rather, with markers for secretory granules and lysosomes [110]. Similarly, in NRK, Vero and HeLa cells, PLD1a and PLD1b do not co-localize with a Golgi marker (mannosidase II), but with a late endosomal and lysosomal marker (lamp-1) [111]. Localization of PLD1-like activity in rat liver lysosomal membranes devoid of mannosidase II was very recently reported [112]. PLD1 thus appears to be localized mainly in organelles and vesicles of the endosomal/lysosomal compartment.

PLD may participate in regulating agonist-induced reorganization of the cytoskeleton. A RhoA-dependent PLD activity (most likely PLD1a or PLD1b) was implicated in lyso-PA-induced formation of stress fibres [113,114]. Two recent studies have reported the association of PLD1 with a detergent-insoluble cytoskeletal fraction. In permeabilized U937 cells, Iyer and Kusner have shown that association of PLD1 with the octyl glucoside-insoluble cytoskeleton is: (i) dependent upon activation by guanosine 5′-[γ-thio]triphosphate (GTP[S]); (ii) accompanied with translocation of ARF and RhoA; and (iii) sensitive to the RhoA-selective *Clostridium botulinum* C3-exotoxin [115]. PLD1 was also found to be localized to a Triton X-100-insoluble cytoskeletal fraction in HL-60 cells [91].

Localization of PLD1 in caveolae has been reported recently [116]. In that study, caveolin-enriched membranes were prepared using a detergent-free method, and may therefore be contaminated with other low-density membrane particles. In contrast, preparations of detergent-insoluble caveolar membranes from multiple cell lines evinced the complete absence of PLD1 immunoreactivity, while a non-ARF-dependent PLD activity was still highly enriched [117]. Immunoblot analyses of caveolar membranes has identified the resident PLD as PLD2 (M. Czarny, unpublished work). Lack of ARF-dependent PLD activity in detergent-insoluble membranes microdomains was reported also by Hodgkin et al. [91]. In addition, in 3T3-L1 adipocytes, PLD2 (but not PLD1) was shown to reside in a low-density membrane fraction that contains the glucose transporter GLUT4 [118]. GLUT4-containing vesicles have previously been shown to co-sediment with caveolae membranes in sucrose density gradients [119]. It may thus be concluded that the PLD activity which is located in caveolae and detergent-insoluble microdomains is PLD2.

Possible functions of PLD1 in vesicular traffic

The Golgi apparatus

ARF isoforms are involved in traffic of transport vesicles in different cell compartments, but most widely studied is the role of ARF1 in the Golgi apparatus [120,121]. ARF1 associates with Golgi membranes upon activation (i.e. exchange of GDP with GTP) and facilitates recruitment of coatomer proteins and budding of transport vesicles from the Golgi. PLD has been implicated in this process by virtue of its being an ARF-dependent enzyme. Evidence in support of a role for PLD includes the following results: (i) ARF-dependent PLD activity is high in Golgi-enriched membranes, compared with other membrane fractions [122]; (ii) Golgi membranes from BFA-resistant PtK1 cells exhibit higher basal PLD activity [122]; (iii) formation of COPI-coated vesicles is inhibited by ethanol [123]; (iv) the ARF1 requirement in coatomer binding can be by-passed by adding exogenous, constitutively active bacterial PLD [123]; (v) primary alcohols inhibit protein transport from the ER to the Golgi, an effect that is reversed by addition of exogenous PA liposomes [124]. While the combined evidence does support a model in which PLD activation and accumulation of PA play a role in recruitment of coatomer to the site of vesicle budding in the Golgi, recent cytofluorescence studies indicate that neither PLD1

[110,111] nor PLD2 [44] are localized in the Golgi. It should also be noted that the activation of PLD may not be an essential part of the basic ARF-mediated vesicle budding machinery in the Golgi of eukaryotic cells, because in yeast such a functionality does not exist. Furthermore, recent work demonstrates that COPI-coated vesicles can be formed *in vitro* from chemically defined synthetic liposomes with reconstituted purified coatomer and ARF1 in the absence of PLD [125,126]. Thus a role for ARF1-dependent PLD1 in budding of COPI-coated Golgi vesicles remains controversial.

TGN

PLD and PA were also implicated in the formation of nascent secretory vesicles in the TGN [127–129]. The role played by PA in the budding of nascent secretory vesicles may be analogous to its proposed role in the Golgi stacks, i.e. to promote recruitment of TGN-specific coat protein(s) such as the AP-1 complex [127]. However, other results have shown that recruitment of AP-1 adaptors on to the TGN is affected neither by exogenously added bacterial PLD nor by addition of neomycin [130].

Endosomes/lysosomes

The latter study has demonstrated that recruitment of AP-2 to endosomes is ARF1-dependent, and that the effect of ARF can be mimicked by the addition of exogenous PLD. Neomycin, which binds phosphoinositides and inhibits PLD activity, was shown to block AP-2 association with endosomes as well as with plasma membranes [130]. Similarly, assembly of AP-2-containing clathrin coats on rat liver lysosomal membranes appears to depend on an ARF/PKC/PIP_2-dependent PLD1-like activity [112]. The process is inhibited by butan-1-ol, but is not affected by t-butanol, and it can be induced by an exogenous bacterial PLD. Interestingly, one function of PLD may involve activation of a type-I PI 4-phosphate 5-kinase, as butan-1-ol completely abolishes PIP_2 synthesis in the lysosomal membranes [112]. Taken together, the localization of PLD1 in membranes of the endosomal/lysosomal compartments, and the data pointing to an involvement of PLD and PA in recruitment of AP-2, strongly implicate PLD1 in ARF-dependent clathrin coat assembly in the endosomal/lysosomal system. It is not yet known whether a similar role is played by PLD1 (or PLD2; see below) in formation of clathrin-coated endocytic vesicles in the plasma membranes.

Secretory vesicles

The localization of PLD1b to secretory granules in RBL-2H3 rat basophilic-leukaemia cells [110] is complemented by the fact that an exocytotic stimulus, i.e. cross-linking of high-affinity IgE receptors, induces the translocation of GFP–PLD1b to the plasma membrane [110]. Similarly, in resting neutrophils, most ARF-dependent PLD activity is co-localized with a secretory-vesicle marker [131]. However, in cells stimulated with formylmethionyl-leucylphenylalanine (fMet-Leu-Phe), ARF-dependent PLD activity is co-localized with the superimposed plasma-membrane and secretory-vesicle markers, suggesting the mobilization of PLD to the plasma membrane [131]. A possible function for PLD as an effector for ARF6 in the exocytic pathway of chromaffin cells was suggested on the basis of results showing the activation of PLD at the plasma membrane by chromaffin-cell secretagogues, concurrent with translocation of ARF6 from the seceretory-granule fraction to the plasma membrane [132]. That study further showed that the activation of PLD correlated in time and in its Ca^{2+}-dependence with the secretory response, and that the latter was attenuated by primary (but not secondary) alcohols [132]. In PC-12 cells, however, primary alcohols do not inhibit the secretory response [133]. Although localization in secretory granules may not be a general property of PLD1 and alcohol-trap experiments have yielded conflicting results, the cumulative data from neutrophils, RBL cells and chromaffin cells seem to suggest that PLD activation could play a role in regulated exocytotic secretion.

MAMMALIAN PLD2

Structural features and comparison with PLD1

The cloning of human PLD1 was followed by the identification of related sequences in expressed sequence tag (EST) databases, including an EST from human fetal liver (R93485) that seemed to encode a distinct PLD homologue [40]. Full-length sequences of this novel PLD, termed PLD2, were soon reported [44,45]. PLD2 is a 933-amino-acid 106 kDa protein that shares a 50–53 % identity with PLD1. PLD2 lacks the 116-amino-acid 'loop' region ($hPLD1a_{505–620}$) that is inserted in PLD1 immediately following the first HKD motif in domain I. Otherwise, PLD1 and PLD2 mainly differ in their N- and C-termini [44,45]. The N-terminal region of PLD2, comprising amino acids 1–308, is not required for catalytic activity, although its removal does confer upon PLD2 a responsiveness to ARF ([92]; see below). Interestingly, insertion of the PLD1 'loop' into PLD2 does not abrogate catalytic activity [92], indicating that, as in PLD1, the N- and C-terminal regions of PLD2 are independent modules that may retain proper interaction regardless of the size of the intervening spacer sequence.

Three splice variants of human PLD2 have been sequenced that represent the full-length PLD2 (hPLD2a), a shorter form lacking 11 amino acids ($hPLD2a_{810–820}$) immediately following the second phosphatidyltransferase motif (hPLD2b), and a truncated form that consists of $hPLD2a_{1–335}$ (hPLD2c) [105]. In addition, that work delineated a putative PH domain in the N-terminal region of PLD1 ($PLD1_{220–329}$) and PLD2 ($PLD2_{203–312}$) [105]. It seems unlikely, however, that this putative PH domain mediates the interaction of PLD1 and PLD2 with PIP_2. Truncation of the entire N-terminal 325 amino acids, including the PH domain, does not abrogate dependence on PIP_2 for activity [50]. Thus the location of the PIP_2-interaction module in PLD2 remains undefined. Like PLD1, PLD2 contains a PX domain in its N-terminal region (amino acids 41–218). PX domains are found in a number of signalling and adaptor proteins, but their function is still poorly defined [52]. The PX domain of PLD2 is located within the region ($PLD2_{1–308}$) whose deletion confers ARF responsiveness to PLD2 (see below), suggesting that the PX domain is not required for catalytic activity, but rather plays a regulatory role.

Tissue distribution and regulation of PLD2 expression

PLD2 seems to be expressed to various extents in almost every tissue and cell type studied. In humans, PLD2 expression was found to be particularly high in the prostate, placenta and thymus, followed by heart, pancreas, kidney and lung [105,134,135]. Low, but measurable, expression was found in most other tissues, with lowest expression observed in skeletal muscle. In rodents, highest expression was found in lung, followed by brain, heart and kidney [45,68]. To date, few studies have examined changes that may occur in PLD2 expression under different physiological conditions. Of note is a recent report showing that granulocytic differentiation of HL-60 cells, induced by dibutyryl cyclic AMP, is accompanied with a very large increase in PLD2 expression, nearly 20-fold over 3 days [70]. On

the other hand, there is no change in PLD2 expression upon differentiation of mouse epidermal keratinocyes induced by 1,25-dihydroxyvitamin D_3 [71].

Regulation of PLD2 activity

In comparison with PLD1, recombinant mouse PLD2 exhibited high basal activity when expressed in insect cells, immunopurified and assayed *in vitro*, as well as when expressed in COS-7 cells and assayed *in vivo* [44]. In contrast, human PLD1 and PLD2 exhibited comparable activities when expressed in insect cells and assayed in the membrane-bound state [135]. These data suggested that PLD2 is regulated by an inhibitory factor that resides in either the cytosol or cell membrane, and that activation of PLD2 may result when the enzyme is relieved of this inhibition. α-Synuclein and β-synuclein were identified as cytosolic proteins that have the capacity to inhibit PLD2 activity *in vitro* [136]. Other proteins that may inhibit PLD2 are PIP_2-binding proteins such as fodrin [137].

However, the view that PLD2 is regulated solely by inhibitory factors may have to be revised. Recent structure–function analysis of PLD2 revealed that PLD2 may be positively regulated by ARF. The intact full-length protein was shown to be mildly sensitive to ARF, exhibiting a 1.5–2-fold activation [92,135]. Removal of most of the non-core N-terminal region, comprising amino acids 1–308, resulted in a protein with much lower basal activity that was stimulated up to 13-fold by ARF proteins, including mammalian ARF1 and ARF5, and yeast ARF2 [92]. These results raise the possibility that PLD2 may constitute at least part of the ARF-stimulated PLD activity observed previously in mammalian cells. The full-length PLD2 may acquire ARF responsiveness through an interaction with another protein and a conformational change that would dislodge the N-terminal region and allow interaction with ARF. Alternatively, PLD2 may acquire ARF responsiveness through the proteolytic removal of its N-terminal region.

Of particular interest is a recent report on the interaction of PLD2 with the epidermal-growth-factor receptor (EGF-R; [138]). That work showed that PLD2 is constitutively associated with EGF-R, and that this association is not affected by treatment with EGF. EGF stimulates the tyrosine phosphorylation of PLD2 on tyrosine-11; however, the tyrosine in this position is absent from the human PLD2, questioning the importance of this phosphorylation. EGF was found to stimulate cellular PLD2 activity but, curiously, the EGF-R-associated activity remained unaffected [138]. Two other receptor tyrosine kinase agonists that activate PLD in an ARF-dependent manner are insulin [84,139] and PDGF [85]. The PLD involved in insulin action is likely to be PLD2, because insulin action is blocked by a catalytically inactive PLD2, but is not affected by a catalytically inactive PLD1 ([93]; see below). Together, the data suggest that receptor tyrosine kinases regulate PLD2 activity, and that PLD2 localization and/or activity are regulated by tyrosine phosphorylation.

Subcellular localization and possible function(s)

When expressed in *S. pombe*, recombinant rat PLD2a was largely associated with cell membranes, but significant activity (about 20%) was found also in cytosol [45]. Similar results were reported for human PLD2 expressed in insect cells [135]. As opposed to PLD1, whose localization in most cells seems to be confined to intracellular membranes, PLD2 was found localized in the plasma membrane in resting REF-52 fibroblasts [44]. Stimulation of these cells with serum resulted in redistribution of PLD2 into 'submembraneous vesicles' that appeared near the plasma-membrane surface and which were suggested to represent endocytic vesicles, perhaps early endosomes [44]. Localization of PLD2 to the plasma membrane under basal conditions is also implied by another study, which showed that PLD2 is associated with the EGF-R in a signal-independent manner [138]. While EGF receptor–PLD2 complex formation is not affected by EGF, phosphorylation of PLD2 on tyrosine-11 is stimulated by EGF [138]. Changes in the subcellular localization of PLD2, which might be induced by EGF, were not examined in that study.

While the above results document the localization of heterologously expressed recombinant PLD2, the localization of endogenous PLD2 was reported recently in terminally differentiated 3T3-L1 adipocytes [118]. PLD1 and PLD2 were localized in a low-density microsomal compartment that was devoid of plasma-membrane markers. Further fractionation of these membranes by sucrose-density-gradient centrifugation revealed that PLD2 was confined to a low-density membrane fraction, while PLD1 was present in higher-density fractions [118]. PLD activity is enriched in low-density detergent-insoluble membrane microdomains that contain the caveolar coat marker proteins caveolin-1 and caveolin-2 [117,140]. Caveolae are plasma membrane 'flask-shaped' invaginations, 50–100 nm in diameter, that are seen in certain cell types, mainly epithelial and mesenchimal cells [141]. Caveolin-1 and caveolin-2 interact with each other and with other proteins, including many signalling proteins [142]. The PLD activity that was found in caveolin-rich membranes in human keratinocytes, human colon and breast-cancer cells, human promonocytic U937 cells and COS-7 cells, is not PLD1 [117]. Rather, immunoblot analysis indicate that it is PLD2 (M. Czarny, unpublished work). The presence of PLD2 in these membrane microdomains is consistent with a plasma-membrane localization. It should be noted that, in 3Y1 rat fibroblasts, PLD1 was reported to be present in caveolae, while the localization of PLD2 has not been examined [116]. The difference between these two studies, with respect to PLD1, appears to be related to the different methods of preparation of caveolin-enriched membranes (M. Czarny, unpublished work).

What role(s) does PLD2 play in cell physiology? Answers to this question are just beginning to emerge. Rat embryo fibroblasts that overexpress PLD2 are morphologically altered, exhibiting numerous cellular projections and some filopodia [44]. Serum stimulation of these cells enhanced the formation of filopodia all around the cell periphery [44]. These data suggest a function for PLD2 in actin cytoskeletal rearrangement. On the other hand, the serum-induced relocalization of PLD2 to an endosomal-like compartment suggests a role in endocytosis [44]. In this context, it is interesting to note that PLD2-Δ1–308 is more responsive to ARF5 than it is to ARF1, and that ARF5 may be involved in endocytosis [130]. Together with data showing that PLD2 is associated with, and phosphorylated by, EGF-R [138], these results suggest that PLD2 might be involved in a receptor-triggered ARF-mediated endocytic process. This speculation is supported by a recent paper of Romero and colleagues [93]. That important work shows that insulin-induced PLD activation and mitogen-activated protein (MAP) kinase stimulation are inhibited by BFA, indicating that these responses are mediated by ARF proteins. Furthermore, it has been demonstrated that both PLD and MAP kinase activation were inhibited by a catalytically inactive mutant of PLD2, whereas an analogous PLD1 mutant was ineffective [93]. BFA blocked also translocation of Raf-1 to the plasma membrane, an event which was reconstituted by the addition of PA. The authors proposed a model according to which insulin stimulates PLD2 activity via an ARF protein, producing PA; the latter participates in formation

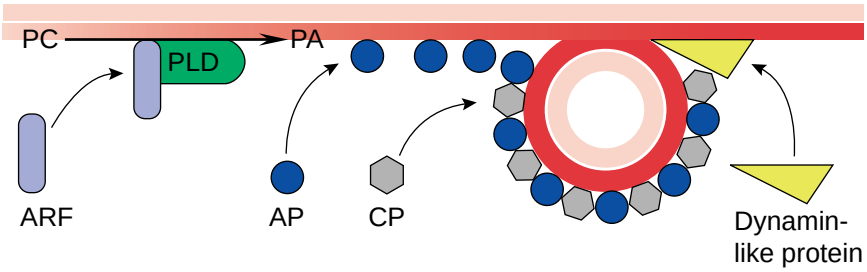

Figure 3 Model of ARF-dependent activation of PLD and its proposed role in vesicle budding

PLD (dark green) has been implicated in formation of: (i) COPI-coated Golgi vesicles [123], AP-1-coated vesicles in the TGN [127], recruitment of AP-2 in endosomes [130] and lysosomes [112], and budding of endocytic vesicles in the plasma membrane [93]. It is generally assumed that stimulation of PLD activity is initiated by recruitment of ARF (light-blue capsules) to the membrane and its activation. It is likely that distinct ARF isoforms are involved in vesicle formation in different compartments. The resultant production of PA by ARF-activated PLD changes the physical properties of the cytosolic face of the membrane (pink). PA may also activate PIP_2 synthesis by activating type I PI 4-phosphate kinases in a positive feedback loop [112,145] (not shown for simplicity). The acidic phospholipids-enriched membrane facilitates recruitment of adaptor proteins [AP (shown as dark-blue circles); where applicable, i.e. in the TGN, endo/lyso-somes and plasma membrane] and coat proteins (CP; grey hexagons). This causes budding of the coated vesicle. The scission of vesicle (red) neck is mediated by dynamin, itself modulated by acidic phospholipids, or a functional analogue of dynamin (lime-green wedge). It is not known whether such an entity is required for COP-coated vesicle budding in the Golgi. It should also be noted that PLD is not essential for vesicle budding in *in vitro* reconstituted systems [125,126]. Effector proteins such as Raf-1 may also be recruited to the PA/PIP_2-rich membrane surface to mediate downstream responses [93].

of endocytic vesicles and in promoting the association of Raf-1 with endosomal membranes, leading to MAP kinase activation [93]. This attractive model is consistent with other studies showing that receptor internalization is required for MAP kinase activation [143] and that Raf-1 is able to interact with PA [144]. It is also consistent with previous suggestions regarding the role played by acidic phospholipids in vesicle budding and/or fusion [145,146]. Finally, this proposed role is analogous to the function envisioned for PLD1 in generation of COPI-coated and AP-2/clathrin-coated vesicles in the Golgi and in the endosomal/lysosomal systems respectively. Figure 3 depicts a model for the proposed role of PLD1 and PLD2 in providing PA as a prerequisite for recruitment of adaptor, coat and effector proteins to budding vesicles, based on the work implicating PLD in formation of coated vesicles in multiple cell compartments.

YEAST SPO14/PLD1

Two distinct PLD activities that were characterized in some detail are present in the budding yeast *Saccharomyces cerevisiae*. One is a Ca^{2+}-independent PLD that, in common with most other PLDs, catalyses both phospholipid hydrolysis and transphosphatidylation [147]. The other is a Ca^{2+}-dependent, non-transphosphatidylating PLD, first identified in yeast strains that lack the Ca^{2+}-independent form [148,149]. The latter enzyme is discussed in greater detail in a subsequent section. The identification and cloning of the gene encoding the yeast Ca^{2+}-independent PLD (*SPO14/PLD1*) provided, for the first time, evidence for an essential function of PLD in eukaryotic cell physiology. Genetic disruption of *SPO14/PLD1* results in a severe sporulation defect [37]. This defect is fully rescued by expression of a catalytically active Spo14p, but not by a catalytically inactive Spo14p mutant [57]. These data indicate that, in yeast, an enzymically active Spo14p is required for completion of meiotic division and sporulation.

Structure and regulation

SPO14/PLD1 encodes a 1683-amino-acid 195 kDa protein that, together with other fungal PLDs from *Candida albicans* and *Schizosaccharomyces pombe*, represent the largest PLD forms so far discovered. Yet, the catalytic core of SPO14/PLD1 (amino acids 694–1129) is among the most compact in eukaryotic PLDs, consisting of 436 residues. As in other PLDs, mutations within the HKD motif (e.g. of the lysine residue) abolish enzyme activity [57,150]. The relatively long N-terminal and C-terminal regions are likely to play an important role in regulation and/or localization. Indeed, it has been demonstrated that the N-terminal 150 amino acids of Spo14p are not essential for its enzymic activity [150]. As in mammalian PLD1 and PLD2, PIP_2 acts as a cofactor for Spo14p PLD activity [35,37], but the site of its interaction with Spo14p has not yet been defined. Spo14p may be regulated by phosphorylation, on one or more serine/threonine residues, that occurs upon its activation during meiosis [151].

Subcellular localization and possible function(s)

The PLD activity of Spo14p is essential, but not sufficient, for sporulation to occur. This has been demonstrated by the failure of an N-terminally-truncated form of Spo14p to rescue the sporulation defect of *spo14/pld1* disruptant strains, although catalytically it is fully active [151]. The missing N-terminal 150 amino acids are important for proper localization of the enzyme to a particulate fraction in mitotically growing cells, and for its relocalization to prospore membranes upon induction of sporulation [151]. Utilizing a fluorescent GFP–Spo14p fusion expressed in yeast, Rudge et al. have followed the changes in the subcellular localization of Spo14p upon induction of sporulation [151]. In pre-induced cells GFP–Spo14p was largely found in the cytoplasm, but 6 h after induction of meiosis the enzyme was found concentrated in intensely fluorescent foci that co-localized with the ends of the spindle, possibly at the spindle pole bodies. At later time points (8–10 h) GFP–Spo14p fluorescence expands to encircle the mature spore membrane [151]. The authors of this important study proposed that, prior to initiation of meiosis, an inactive Spo14p is localized in a cytoplasmic, possibly cytoskeletal, compartment. Subsequently, Spo14p is translocated to the spindle pole bodies and then to spore membranes, where it has access to its phospholipid substrate PC and cofactor PIP_2. However, the precise function of Spo14p in this locale is not yet understood.

The fact that *spo14/pld1* yeast are viable and exhibit no growth defect under vegetative growth conditions has indicated that Spo14p is not an essential component in membrane traffic

mechanisms. In fact, unlike mammalian PLDs, the intact Spo14p is not regulated by yeast ARFs *in vitro* [150]. Nevertheless, recent work has found an essential function for Spo14p in the secretory pathway, under specific physiological conditions [152,153]. *SEC14* encodes a yeast PI/PC transfer protein that is essential for normal membrane transport from the Golgi [154]. The Sec14p requirement may be bypassed by inactivating mutations in several genes, including, *CKI1, CCT1* and *CPT1* (all of which encode enzymes of the CDP-choline pathway for PC biosynthesis) [155]. Another *sec14* by-pass gene is *SAC1* [156], recently identified as a novel phosphoinositide phosphatase [157]. Intact Spo14p activity is essential for by-pass of a $sec14^{ts}$ secretion defect by a mutant *cki1* (choline kinase) [152]. Importantly, Spo14p PLD activity is elevated in strains carrying the $sec14^{ts}$ mutation, raising the possibility that Spo14p is under Sec14p control [152]. The essential requirement for an intact Spo14p in effecting bypass of $sec14^{ts}$ secretory defect was demonstrated also for other bypass mutants, including *sac1* [153]. Together, these data demonstrate that elevated production of PA by Spo14p is a necessary, but not sufficient, condition for by-passing the secretory defect caused by mutation of *SEC14*, regardless of the specific by-pass mechanism. This indicates that a tight functional link may exist between Sec14p and Spo14p, the exact nature of which remains to be elucidated.

PLANT PHOSPHOLIPASE D GENES

While the main focus of the present review is on mammalian and yeast PLDs, plant PLD research has continued to provide important insights into the regulation and function of the enzyme that are likely to be relevant to other eukaryotic PLDs. Three distinct PLD genes that encode functionally different proteins were cloned from various plants (PLDα) and from *Arabidopsis thaliana* (thale cress; PLDβ and PLDγ) (reviewed in [47]). The three plant genes share an N-terminal C2 domain, involved in Ca^{2+}-dependent phospholipid binding [53], which is absent from cloned mammalian and yeast PLD genes. Indeed, all plant PLDs are stimulated by Ca^{2+}, although PLDβ and PLDγ are stimulated at micromolar concentrations, whereas PLDα requires millimolar concentrations [48]. Another difference among plant PLDs is in their dependence on phosphoinositides, exhibited by PLDβ and PLDγ, but not by PLDα. As in other C2-containing proteins, one mode of regulation of plant PLDs may involve a Ca^{2+}-dependent translocation to a membrane compartment, demonstrated upon wounding of castor-bean leaves [158]. Recent studies indicate that, in *Arabidopsis*, the three isoforms exhibit different subcellular distribution and tissue expression patterns [159] and have an isoform-specific substrate preference that depends on the composition of the substrate liposomes [160]. The function of plant PLDα was addressed in a study in which its expression was suppressed by introducing a PLDα antisense cDNA into the *Arabidopsis* genome. This had no effect on normal plant growth and development, but it significantly retarded senescence of leaves treated with the phytohormones abscisic acid and ethylene [161]. As both hormones induce PLDα expression in normal leaves, these data indicate that the induction of PLDα mediates hormone-induced senescence in plants [161]. The reader is referred to a recent review for a more detailed discussion of the structure, regulation and function of plant PLDs [162].

OTHER EUKARYOTIC PLD ACTIVITIES

Oleate-activated PLD

Long thought to exist exclusively in plants, mammalian PLD activity was first discovered in brain membranes by Kanfer and colleagues [163,164]. The activity was cryptic unless a surfactant such as sodium taurodeoxycholate was present in the assay. Because it is activated by sodium oleate and other unsaturated fatty acids [165], the enzyme is usually referred to as oleate-activated PLD. The properties of oleate-activated PLD have recently been reviewed [166]. Like other PLDs, oleate-activated PLD catalyses the formation of phosphatidylalcohols via transphosphatidylation [167]. The enzyme exhibits a clear substrate preference for PC [168–170], which is particularly pronounced with endogenous membrane phospholipid substrates [171]. Oleate-activated PLD is an integral membrane enzyme that requires a detergent for its solubilization [168,172]. Over the years it has been detected in different cellular compartments, including plasma membrane, endoplasmic reticulum and nucleus [108]. The purification of porcine lung oleate-activated PLD to homogeneity, reported a few years ago, yielded a protein band of 190 kDa [30]. While this molecular mass is close to previous estimates [168], the enzyme has not yet been cloned. On the basis of its ability to catalyse a transphophatidylation and its preference for PC, it may be speculated that oleate-activated PLD will turn out to be a member of the known PLD gene superfamily.

The regulation and function of oleate-activated PLD are rather obscure. Until recently, there was no evidence that non-esterified fatty acids have any physiological role in its activation *in situ*. However, an exciting recent paper has demonstrated that oleate-activated PLD is the major form detected *in vitro* in lysates of Jurkat T cells [173]. Phosphatidylbutanol formation in intact cells, as well as *in vitro* PLD activity, are both dramatically elevated upon induction of apoptosis [173]. This is accompanied with loss of oleate-responsiveness of the enzyme *in vitro* and with elevated levels of non-esterified fatty acids in the cells [173]. The results are consistent with a model in which induction of apoptosis elevates cellular non-esterified fatty acids, which then activate oleate-activated PLD. In another study, the level of oleate-activated PLD in cardiac muscle was shown to decrease upon induction of diabetes [174]. Conversely, increased activity was found in human breast-cancer tissue [175]. Together, these data suggest that oleate-activated PLD is subject to both acute and chronic regulation in certain mammalian cells. Further advances in our understanding of this enzyme critically depend on cloning of the gene that encodes it.

Phosphoinositide-specific PLDs

Hydrolysis of phosphatidylinositol (PI) to PA in intact human neutrophils was in fact the first indication of a signal-activated PLD in mammalian cells [3]. Although most subsequent studies focused on PC hydrolysis, there were several early reports of PI-hydrolysing PLD (PI-PLD) activities. PI-PLD activities were identified in human neutrophils [176,177], bovine lung [178], Madin–Darby canine kidney cells [179] and more recently in B lymphocytes [180]. Two common features of these enzymes were their apparent localization in cytosol and their stimulation by Ca^{2+}. Whereas other PLDs (e.g. oleate-activated PLD) are highly specific for PC as substrate, cytosolic PI-PLDs appear to be more permissive in their interaction with phospholipid substrates, and were reported to utilize PI and PE [179], PI and PC [177] or PE, PC and PI [178] in that order of preference. Whether these differences are due to the different assay conditions utilized in these studies, or represent genuine differences in their substrate specificity which reflects a molecular heterogeneity, is still not known. Very recently, three predominantly cytosolic PLD activities were partially purified, termed PI-PLDa, PI-PLDb and PI-PLDc [181]. These PI-PLDs were shown to hydrolyse D-3-polyphosphoinositides, preferentially PI 3,4,5-trisphosphate

(PIP_3; PI-PLDa and PI-PLDb) and PI 3-phosphate (PI-PLDc) [181]. It is tempting to speculate that these new PI-PLDs are related to the previously described activities, as they all are dependent on Ca^{2+}. The discovery of PLD activities that degrade D-3-polyphosphoinositides is of great importance, in view of the established function of these PI 3-kinase products in signal transduction and intracellular membrane traffic [182]. Thus one function of the newly discovered PI-PLDs might be to turn off PIP_3-mediated signalling. The product of this hydrolysis is a novel water-soluble inositol phosphate, D-*myo*-inositol 3,4,5-trisphosphate, which could have signalling functions of its own.

Yeast Ca^{2+}-dependent PS/PE-hydrolysing PLD

The genetic disruption of *SPO14/PLD1* helped uncover a second yeast PLD activity that proved to be quite different from previously characterized PLDs [148,149]. In our laboratory, the discovery of yeast PLD2 was coincidental and occurred as we began to assay PLD activity in *spo14/pld1* disruptant strains in the absence of EGTA and EDTA. This has revealed a major Ca^{2+}-dependent PLD activity with some unusual properties. First, the enzyme has a strong preference for aminophospholipids as substrates (PE = PS $\gg$ PG > PC); it has no lysophospholipase nor glycerophosphodiesterase activity [148]. Secondly, unlike yeast Spo14p and other PLDs, the yeast PLD2 does not require PIP_2 as a cofactor. Third, the yeast PLD2 does not catalyse a transphosphatidylation reaction with the usual primary short-chain alcohols. (However, the possibility cannot be excluded that some specific acceptor molecules(s) will be discovered.) These properties suggested that yeast PLD2 is likely to differ significantly from other PLD members of the HKD/phosphatidyltransferase gene family. This was affirmed as the sequencing of the entire yeast genome was completed, revealing that *SPO14/PLD1* is the only PLD member of this family in the entire yeast genome. It may thus be speculated that yeast PLD2 is a member of a different PLD gene family. Confirmation of this speculation must await the cloning of yeast PLD2.

Mitochondrial PE-PLD

Recent work has characterized a mammalian mitochondrial PLD-like activity [183,184]. This enzyme was identified in rat enterocyte mitochondria and its activity monitored by measuring PA and headgroup production from endogenous mitochondrial phospholipids. The rat enterocyte mitochondrial PLD activity seems to be related to an activity previously described in rat liver mitochondria [185]. The results suggest that Ca^{2+} stimulates the hydrolysis of PE by a PLD-like activity that does not catalyse a transphosphatidylation reaction. In addition, the mitochondrial PLD is stimulated by oxygen free radicals [184] and polyamines [186]. The purity of the mitochondrial membrane preparation has not been reported; more importantly, the results have not yet been confirmed with an exogenous phospholipid substrate. Nevertheless, the possible existence of this putative mitochondrial PLD is of interest, because some of its properties are reminiscent of the recently characterized Ca^{2+}-dependent, PE-hydrolysing, non-transphosphatidylating yeast PLD2 described above.

N-acyl-PE-PLD

A PLD-type phosphodiesterase that catalyses the hydrolysis of *N*-acyl-PE to phosphatidic acid and free *N*-acylethanolamine was identified in dog brain microsomes [187]. While so far it has not been shown to play a role in signal transduction, the *N*-acyl-PE-specific PLD may be involved in producing anandamide (i.e. *N*-arachidonoylethanolamine) and anandamide-like molecules [188]. The properties and regulation of this enzyme have been reviewed recently [189,190], and a role for *N*-acyl-PE and its PLD degradation products in glutamate-induced excitotoxicity was proposed [190].

Lyso-PLD

Relatively little studied, but of great potential interest, are a number of PLD-type enzymes that utilize lysophospholipid substrates. A Mg^{2+}-dependent lyso-PLD was first identified in rat brain microsomes and subsequently in microsomes from other tissues [191]. A distinct, Ca^{2+}-dependent lyso-PLD was found in microsomes from rabbit kidney medulla [192]. Both enzymes specifically hydrolyse ether-linked lyso-PC and lyso-PE. The properties of these enzymes and their possible roles in phosphoacylglycerol metabolism have been reviewed [193]. These enzymes may be related to a lyso-PLD activity from microsomes of rat intestinal epithelial cells that was implicated in the metabolism of platelet-activating factor [194]. Distinct from tissue lyso-PLD, rat plasma contains a lyso-PLD activity that exhibits a different metal-ion requirement (preference for Co^{2+} and Zn^{2+}) and a different substrate specificity [195,196]. To date, none of these enzymes has been purified or cloned. Furthermore, so far there has been no evidence for lyso-PLD activation by extracellular stimuli. The product of lyso-PLD is lyso-PA, a multifunctional extracellular hormone whose mechanisms of formation are not entirely clear [197]. Lyso-PLDs may well contribute to generation of lyso-PA in the body, but confirmation of this speculation is likely to require much additional research.

GPI anchor-specific PLD (GPI-PLD)

GPI-PLD was discovered [198] as a serum enzyme that specifically hydrolyses the inositol-phosphate linkage in proteins (e.g. acetylcholinesterase) that are anchored to the cell membrane by a GPI moiety (see [199] for a review). The enzyme was cloned [34], but it has no relationship to the PC-hydrolysing PLD genes that are the main subject of the present review. The reader is referred to a recent review that focuses on the role of GPI-anchor hydrolysis in signal transduction [200].

INVOLVEMENT OF PLD IN MEDIATING AGONIST-INDUCED CELL RESPONSES

In the absence of selective and potent inhibitors of PLD, experimental approaches that have been employed for implicating PLD in a specific cell function are few in number and limited in power. Foremost, an inhibitory effect of primary alcohols on a given response is often interpreted as evidence for an involvement of PLD. Such an interpretation is valid if the data demonstrate the reduction of signal-induced PA and the ineffectiveness of secondary alcohols, which should always serve as a negative control. Another approach has been to mimic agonist action by exogenously added PA, or by treatment with an exogenous (usually bacterial) PLD. This approach is more powerful when implemented in a cell-free assay that reconstitutes the response studied, because cell-surface actions of PA and PLD are difficult to interpret. Propranolol, an inhibitor of PA phosphohydrolase, elevates endogenous PA by preventing its degradation, and has been utilized to mimic agonist action as well as to resolve the relative contribution of PA versus DAG in a given response. The pharmacological and biochemical approaches can now be complemented with molecular ones, such as overexpression of wild-type and dominant-negative PLD cDNAs, although the latter have yet to be widely employed. The utilization of various experimental approaches is exemplified in

the following paragraphs, where some prominent cell responses in which PLD has been implicated are reviewed.

The case of the human neutrophil: activation of NADPH oxidase and degranulation

The activation of PLD in human (and other) neutrophils by fMet-Leu-Phe and other stimulants has been very extensively studied (see Table 1). Therefore, it is perhaps not surprising that the role of PLD and PA in agonist-mediated cellular events has been most closely examined, and is probably best established, in this cell type. To summarize early work, a correlation was found between PLD activation, PA mass accumulation and NADPH oxidase activation in f-Met-Leu-Phe-stimulated neutrophils. Secondly, primary alcohols were found to inhibit NADPH oxidase activation, whereas propranolol (which blocks PA conversion into DAG) potentiated the response. Thirdly, PA directly stimulated NADPH oxidase activity *in vitro*. A similar set of results has implicated PLD and PA also in release of neutrophil granule content. These studies were reviewed elsewhere [201,202]. Despite all this progress, the identity of the PLD that is activated in neutrophils is still unknown, although it is highly likely to be PLD1. The mechanism of PA action in stimulating NADPH oxidase activity is not fully understood. PA induces activation of one or more protein kinases that regulate NADPH oxidase activation in a cell-free system, as evinced by inhibitory actions of kinase inhibitors and depletion of ATP [203]. McPhail and colleagues have further characterized the PA-depenedent kinase as a cytosolic 125 kDa protein that is widely expressed, and that appears to have a dual, serine and tyrosine specificity on $p47^{phox}$ [204]. Additional actions of PA are, however, likely.

Synthesis and release of matrix metalloproteases (MMPs)

MMPs play an important role in degrading basement membrane and thereby facilitating invasion and metastasis of cancer cells. PLD seems to be involved in mediating MMP-2 release, in response to ligation of human-cancer cell-surface receptors by laminin. This was suggested on the basis of (i) the activation of PLD by laminin, (ii) stimulation of MMP-2 release by exogenously added PA, and (iii) inhibition of laminin-induced MMP-2 release by primary, but not secondary, alcohols [205]. Furthermore, photolysis of caged-PA induced MMP-2 release in these cells, thus mimicking the action of laminin [206]. PLD also is likely to mediate the synthesis and release of MMP-9 in response to phorbol-ester stimulation. In that study, a short-chain PA analogue mimicked phorbol-ester-induced release of MMP-9, whereas propan-1-ol attenuated it [207]. What remains to be determined is whether PLD is involved in this process in the capacity of a signalling molecule (i.e. an upstream regulatory element) or as part of the protein-transport machinery. Identification of the PLD isoform involved may help resolve this dilemma. In this context it is interesting to note that activation of PLD and production of PA modulate integrin-mediated adhesion in eosinophils [208]. Hence, another mechanism through which PLD may affect laminin-induced release of MMPs is by 'inside-out' signalling directed at cell-surface integrins.

Provision of choline for acetylcholine synthesis in cholinergic neurons

Cholinergic neurons express a constitutively active PLD, that provides choline which is utilized for acetylcholine synthesis [209]. This enzyme may not be expressed in all cell types, as PLD appears not to be involved in the basal turnover of phospholipids in some cells [210]. The identity of this constitutively active PLD, whether its expression is restricted to cholinergic neurons, and the mode of its regulation are still unknown. Nevertheless, its important function is suggested by the fact that prenatal choline supplementation increases hippocampal PLD activity [211]. This dietary manipulation is known to cause a persistent modification in postnatal brain choline metabolism and is associated with improved visuospatial memory [212].

Other PLD-mediated cell responses

As discussed above, PLD1 and PLD2 have been suggested to play a role in regulating the cytoskeleton and are assumed to act at multiple sites in vesicular transport and cell-signalling pathways. In addition, PLD has been implicated in mobilization of arachidonic acid and, in some cases, stimulation of prostaglandin synthesis, in numerous cell types ([213] and citations therein). Similarly, a large body of evidence implicates PLD in regulating the activity of cyclic nucleotide phosphodiesterase, probably via a direct action of PA on rolipram-sensitive phosphodiesterase type 4 ([214] and citations therein). Other functions of the cloned PLD gene products, and of the yet-to-be-cloned PLD activities, will no doubt be discovered in the future.

CONCLUDING REMARKS

The cloning of eukaryotic PLDs from plant, fungal and animal sources was a breakthrough that enabled much of the rapid advances in PLD research that we have witnessed in the last 5 years. Yet more surprises are probably in store as the structure of additional PLDs will be elucidated. An important and quantitatively major PLD that has yet to be molecularly cloned is the oleate-activated PLD. Likely a member of the known HKD/phosphatidyltransferase gene family, so far it has eluded identification. A PLD that may belong in another, novel, PLD gene family, is the yeast Ca^{2+}-dependent PE/PS-PLD. Cloning these genes will surely provide new insights into the regulation of and the catalytic mechanisms of the PLD reaction.

The cloned PLDs are enjoying unprecedented attention, with much of the research focused on the interrelated questions of their regulation and localization. The subcelluar localization of the different PLD isoenzymes is being addressed using immunological and molecular-biological tools. These first studies will certainly be followed by many more. Hence, a fairly complete and hopefully coherent picture of PLD localization in different cell types and under different physiological conditions may realistically be expected in the near future.

The most challenging and important problem involves the elucidation of the functions of different PLDs in their various locales. Immunological and molecular tools will be invaluable in establishing these functions in cultured cells. Insights into the essential physiological roles played by mammalian PLDs will hopefully be gleaned from transgenic PLD-knockout-animal models, an advance that is eagerly awaited since the first cloning of mammalian PLD1 and PLD2. Together, progress in these two fields will provide a fuller understanding of the downstream events that occur in consequence of PLD activation and, in particular, the identity of target proteins that are directly regulated by PA. Recent evidence suggests that PA interacts with protein kinases and protein phosphatases that participate in various signal-transduction cascades. We are thus faced with the daunting, yet exhilarating, prospect that, following the elucidation of PLD upstream regulatory mechanisms and downstream effector responses, PLD researchers will still have to work out details of the cross-talk of PLD pathways with the complex

signal-transduction networks that determines specific cellular responses to different environmental challenges.

We thank Yona Eli and Tovi Harel-Orbital for excellent technical assistance. We are very grateful to the anonymous reviewers for correcting errors and omissions. Research in M.L.'s laboratory was supported in part by grants from the Israel Science Foundation (Jerusalem), the U.S.–Israel Binational Science Foundation (Jerusalem), the Minerva Foundation (Munich) and the Forchheimer Center for Molecular Genetics (Rehovot). M.L. is the incumbent of the Harold L. Korda Professorial Chair in Biology. M.C. was supported by a FEBS Long-term Fellowship.

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