Mutagenesis of phospholipase D defines a superfamily including a *trans*-Golgi viral protein required for poxvirus pathogenicity

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Phospholipase D (PLD) genes are members of a superfamily that is defined by several highly conserved motifs. PLD in mammals has been proposed to play a role in membrane vesicular trafficking and signal transduction. Using site-directed mutagenesis, 25 point mutants have been made in human PLD1 (hPLD1) and characterized. We find that a motif (HxKxxxxD) and a serine/threonine conserved in all members of the PLD superfamily are critical for PLD biochemical activity, suggesting a possible catalytic mechanism. Functional analysis of catalytically inactive point mutants for yeast PLD demonstrates that the meiotic phenotype ensuing from PLD deficiency in yeast derives from a loss of enzymatic activity. Finally, mutation of an HxKxxxxD motif found in a vaccinia viral protein expressed in the Golgi complex results in loss of efficient vaccinia virus cell-to-cell spreading, implicating the viral protein as a member of the superfamily and suggesting that it encodes a lipid modifying or binding activity. The results suggest that vaccinia virus and hPLD1 may act through analogous mechanisms to effect viral cellular egress and vesicular trafficking, respectively.

Keywords: phospholipase D (PLD)/SPO14/vaccinia virus/VP37

Introduction

The initiation of signal transduction cascades through G-protein-coupled receptors or tyrosine kinase receptors results in the activation of membrane-associated lipid-modifying enzymes, including the phospholipases A2, C and D (reviewed in Divecha and Irvine, 1995). More directly, phospholipase D1 (PLD1) can be activated *in vitro* by protein kinase C (PKC) and members of the ARF and Rho small G-protein families, suggesting that its activation during agonist stimulation ensues from the controlled regulation of these effectors (Hammond *et al.*, 1997). Phospholipase D (PLD) catalyzes the hydrolysis of phosphatidylcholine (PC) to yield phosphatidic acid (PA)

and choline (reviewed in Exton, 1994). Several cellular functions have been proposed for PLD, including critical roles in regulation of secretion, reorganization of the actin cytoskeleton and control of cell proliferation, but all of the hypotheses remain unproven (reviewed in Klein *et al.*, 1995). Until recently, a major factor hindering progress in this field was the absence of molecular tools.

A breakthrough was made with the cloning of a plant PLD cDNA (Wang *et al.*, 1994). The properties of the plant PLD are distinct from mammalian PLD at a biochemical level (Brown *et al.*, 1995). However, the plant protein contained novel motifs that were sufficiently well conserved across a broad phylogenetic range to identify a yeast gene required for meiosis as a PLD (Rose *et al.*, 1995), and to provide an entry into the cloning of mammalian PLD cDNAs (Hammond *et al.*, 1995; Colley *et al.*, 1997). Scrutiny of the mammalian PLD1 cDNA revealed none of the motifs that might have been expected by analogy to phospholipase C, such as SH2, SH3 or PH domains (Hammond *et al.*, 1995). In addition, PLD1 does not encode sequences previously shown to interact with RhoA (Reid *et al.*, 1996). However, database searches using mammalian PLD1 revealed the existence of PLD homologs in numerous species (Hammond *et al.*, 1995) and defined four regions of highly conserved sequence (Figure 1; reviewed in Morris *et al.*, 1996). The most prominent sequence contains an invariant charged motif, HxKxxxxD, which was proposed to mediate catalysis (Hammond *et al.*, 1995). Two separated copies of this motif, embedded in surrounding sequences that are also conserved but less rigorously duplicated, are found in all PLD homologs (Morris *et al.*, 1996).

This 'HKD' motif is also found in two phospholipid synthesis enzymes, cardiolipin synthase (CLS) and phosphatidyl serine synthase (PSS) (reviewed in Morris *et al.*, 1996) and in a pair of proteins encoded by the Poxviridae family of viruses (reviewed in Koonin, 1996; Ponting and Kerr, 1996). One of these proteins, VP37, which contains only a single, partially conserved form of the HKD motif, is required for efficient cell-to-cell spreading by vaccinia virus and has been studied extensively. Although its mechanism of action is unknown, it is expressed in the Golgi complex and plays a role in the wrapping of viral particles in *trans*-Golgi membranes and their subsequent transit from the cell (Hiller and Weber, 1985). This prompts the hypothesis that VP37 and mammalian PLD1 are homologous in a functional sense, since PLD1 is also expressed in the Golgi complex (Colley *et al.*, 1997) and has been proposed to regulate vesicular budding (reviewed in Bednarek *et al.*, 1996).

In this study, we have used site-directed mutagenesis and a combination of *in vitro* and *in vivo* systems to determine whether the HKD motif and other conserved sequences are required by the PLD superfamily. Our

Fig. 1. Sequence alignment of the PLD superfamily. Solid line, human PLD1. Boxes, location of regions of highly conserved sequence. Shaded regions, invariantly conserved amino acids. Asterisks, sites of amino acids mutated in the current study. Arrow, critical serine or threonine residue. See text for details. Δ , *Arabidopsis* PLD contains an insertion of 12 amino acids at this site. Abbreviations: hPLD1, human PLD1; mPLD2, mouse PLD2; cePLD, *Caenorhabditis elegans* PLD; scPLD, yeast PLD (SPO14; *Saccharomyces cerevisiae*); atPLD, *Arabidopsis thaliana* PLD; bsPLD, bacterial (*Bacillus subtilis*) PLD; ecCLS, *Escherichia coli* cardiolipin synthase; ecPSS, *E. coli* phosphatidylserine synthase; vvK4, vaccinia virus K4; vvVP37, vaccinia virus P37.

findings support the importance of the HKD motif and a conserved serine but reveal that several other uniformly conserved sequences are less critical, or even seemingly do not contribute to PLD function. We propose a mechanism wherein the conserved serine undergoes a covalent reaction generating a PLD-PA intermediate. Despite very limited homology to PLD, VP37 functionality is shown to depend on the integrity of the HKD site. This result suggests that a search for inhibitors of the presumed VP37 enzymatic or lipid-binding activity might lead to new agents for anti-poxvirus therapy.

Results

A lysine that is invariant in all PLD homologs is required for catalysis by mammalian PLDs

To investigate specific conserved amino acids, we employed site-directed mutagenesis. Although extremely valuable, one caveat with this approach is that even single amino acid changes can induce protein instability or misfolding. Thus, trivial reasons for loss of function need to be ruled out. Such controls were carried out for all mutants and are presented here in detail for hPLD1(K898R).

K898 is found in the second HKD motif of hPLD1 (Figure 1, region IV) and is invariant in all PLD homologs

(including CLS, PSS and the viral proteins K4 and VP37). Our initial mutation involved making the most conservative substitution possible, changing it to arginine (K898R). This substitution retains the basic charge of the lysine side group, but changes its orientation and distance from the protein backbone. To assess enzyme activity, the mutated cDNA was expressed in COS-7 cells under the control of a CMV promoter, after which the cells were lysed and assayed for PLD activity in the presence of ARF1 (which is required to activate PLD1) using a standard *in vitro* head group release assay (Hammond *et al.*, 1995). The level of activity was compared with that found in cells expressing either a control protein that exhibits no PLD activity (Gbx-2) or wild-type hPLD1 (Figure 2A). The results demonstrate that cells transfected with hPLD1(K898R) do not contain PLD activity above background levels.

To address whether activity was lost because the mutant protein is unstable, Western blot analyses were performed using a monoclonal antibody to detect the Flu-epitope tag fused to the recombinant proteins. hPLD1(K898R) was found to be expressed as effectively as wild-type hPLD1, ruling out protein instability (Figure 2B). Similar results were observed for mouse PLD2 (mPLD2) and an equivalent mutant (K758R; see Figure 1). Substitution of arginine for lysine at amino acid 758 (aa758) resulted in mPLD2

Fig. 2. A PLD mutant in the HKD motif is catalytically inactive. (A) hPLD1 encoding a substitution of arginine for lysine at aa898 was transfected into COS-7 cells and examined for activity in comparison to wild-type PLD1 and a control vector. Wild-type PLD1 is largely inactive unless stimulated by an effector such as ARF1. A small increase is observed for the control vector (Gbx-2, a homeobox gene that does not encode PLD activity) in the presence of ARF1 due to activation of endogenous COS-7 cell PLD present in the cell extract. No additional activity over the control is observed for hPLD1(K898R). (B) Western analysis of transfected cells. Aliquots of cells transfected with a control expression plasmid (Gbx-2; a 50 kDa protein), the expression plasmid lacking any inserted cDNA (pCGN) or wild-type or mutant PLD1 or PLD2 cDNAs, were analyzed by Western blotting using an anti-Flu tag monoclonal antibody. Mutant PLD1 and PLD2 are expressed at wild-type levels, demonstrating that the proteins were not destabilized by the K→R mutation. The slight difference seen here between wild-type and mutant PLD1 was not observed reproducibly. 5'Δ-PLD1 and 5'Δ-PLD2 constitute PLD structural mutants lacking ~300 aa at the amino-terminus. They are shown here to further illustrate the specificity of the bands identified for the full length wild-type and mutant PLD proteins. PLD1 exhibits heterogeneity with a major band migrating at 122 kDa and two minor slightly larger bands. PLD2 (108 kDa) breakdown products are frequently observed.

losing all measurable activity *in vitro* (data not shown), although the mutant protein is fully stable.

Another possibility was that the mutation induced altered PLD activity specifically in the *in vitro* assay. Although well accepted, the *in vitro* assay is not physiological in several respects. To address this question, we quantified PLD activity *in vivo* using the transphosphatidyl assay (Wakelam *et al.*, 1995). This method measures production of phosphatidylalcohol, which is formed by PLD when alcohol is used as a nucleophile instead of water to cleave PC (see Figure 8 and Discussion). To determine mutant PLD activity *in vivo*, cells were transfected with the expression constructs and assayed for phosphatidylbutanol (PBut) accumulation (Figure 3). As reported previously (Colley *et al.*, 1997), cells expressing wild-type PLD1 or control plasmids exhibit little PLD activity in the basal state (Figure 3, first four lanes), whereas COS-7 cells expressing PLD2 manifest high levels of PLD activity (Figure 3, lane 5). An ~200-fold increase in PBut accumulation is observed (data not shown). In contrast, COS-7 cells expressing mPLD2-(K758R) do not exhibit increased levels of PLD activity over background (Figure 3, lane 6), providing a striking demonstration that its lack of activity *in vitro* accurately reflects its behavior *in vivo*.

Stimulation of cells with phorbol ester results in the activation of several protein kinase C isoforms including PKCα, which then activate endogenous PLD in control cells (Figure 3, lanes 7 and 9) and both endogenous and

Fig. 3. PLD mutants are catalytically inactive *in vivo*. A transphosphatidyl assay was performed to examine the behavior *in vivo* of mutated PLD proteins. Accumulation of phosphatidylbutanol (PtdBut) in COS-7 cells transfected with control and wild-type or mutant PLD1 and PLD2 expression plasmids was determined. Some culture dishes were additionally stimulated with 100 nM PMA during the 30 min assay period. Each lane represents an individual dish of cells. The experiment was repeated several times and a representative result is shown.

recombinant PLD1 in the PLD1-transfected cells (Figure 3, lane 8). An ~4-fold increase in PBut accumulation is observed over PMA-stimulated control cells (data not shown). However, cells expressing hPLD1(K898R) do not exhibit increased PLD activity over background (Figure 3, lane 10), again confirming that the mutant PLDs are inactive *in vivo*.

In the absence of structural information, it is not possible to predict which mutations will cause problems in folding. However, this can be addressed using approaches such as the two-hybrid assay. Two-hybrid interactions are highly

Fig. 4. RhoA and hPLD1 interactions in the two-hybrid assay. Yeast strains were constructed harboring either a carboxy-terminal fragment of PLD1 fused to the LexA DNA binding domain or RhoA fused to the VP16 activation domain. None of the PLD1 or RhoA fusion proteins activated the reporter gene *LacZ* when expressed alone (lanes 1–4). When combined through mating of the respective yeast strains, PLD1 was observed to interact with a dominant active form of RhoA (RhoA-valine14), but not with wild-type RhoA (lanes 5 and 6). PLD1(K898R), when combined with RhoA-V14, activated the reporter plasmid almost as well as wild-type PLD1 (lane 7). Data points represent duplicate samples. Other controls demonstrating the specificity of the RhoA–PLD1 interaction were performed but are not shown (Y.Zhang and M.A.Frohman, unpublished).

sensitive to changes that result in the misfolding of target proteins. Separate experiments have revealed that an activated allele of RhoA (RhoA-valine 14) interacts with PLD1's carboxy-terminus (Y.Zhang and M.A.Frohman, unpublished). To examine the consequence of the K898R mutation on PLD1's interaction with RhoA, the corresponding carboxy-terminus fragment of the mutant was subcloned into the appropriate two-hybrid vector as a fusion protein and tested (Figure 4). The results indicate that the mutant PLD1 peptide interacts with RhoA efficiently, making it unlikely that the mutation induced significant conformational changes in PLD1.

In addition, hPLD1(K898R) was expressed in baculovirus. The recombinant protein is stable, electrophoreses with the correct apparent mobility and is completely inactive in the presence of ARF1 (as well as other known PLD activators such as RhoA and PKC; data not shown). Subcellular localization was also examined. PLD1 localizes to a peri-nuclear region consistent with Golgi, endoplasmic reticulum and late endosomes (Colley *et al.*, 1997). hPLD1(K898R) localized similarly, suggesting that the mutated PLD1 is membrane-associated and correctly targeted (data not shown).

Taken together, these results suggest that K898 is

required for PLD1-mediated catalysis of PC and that the loss of enzymatic activity is not due to protein instability, misfolding or mislocalization.

Site-directed mutagenesis identifies critical invariant amino acids

A series of amino acids were mutated. The mutant PLDs were then expressed in COS-7 cells and analyzed as described above (Figure 5). Two mutant proteins were unstable and could not be characterized (see legend to Figure 5). Several others failed to interact with RhoA in the two-hybrid assay and might exhibit some degree of structural alteration (see legend to Figure 5). None of the stable mutant proteins displayed any alteration in subcellular localization (data not shown). All protein activities tested using transphosphatidylase assays yielded results similar to those generated through *in vitro* analyses (data not shown). Finally, no differences in relative activity were observed through activating PLD using other types of effectors, such as RhoA, Rac1 or PKC (Table I).

HKD motifs. A series of conservative and non-conservative substitutions were made. Without exception, mutation of any amino acid in either HKD motif resulted in complete or virtually complete loss of enzymatic activity. This finding supports predictions that the motif is critical for PLD catalytic function (Hammond *et al.*, 1995; Morris *et al.*, 1996; Ponting and Kerr, 1996). These results distinguish between two existing models for catalysis: one model postulated that each motif would function independently, whereas the other proposed that each motif would constitute half of the actual active site (Ponting and Kerr, 1996). Since single point mutations in either motif abolish activity completely, our results demonstrate that the duplicated motifs do not function independently.

GGGR/GSRS motif. Four of the seven substitutions resulted in reduced but significant levels of catalysis. The results suggest that the small non-polar amino acids (glycines) are important but not as critical as the charged HKD motifs and may effect correct positioning of other requisite amino acids, since in some cases, double mutations 'rescued' single mutations that inhibited catalysis [e.g. Table I: G479A (1% residual activity in the presence of ARF) as compared with G478A-G479A (21% residual activity)].

Contrasting results were observed with serine 911 (S911), which is highly conserved (as either a serine or threonine) in the second HKD motif but is not present in the first. Mutation of S911 to threonine resulted in retention of significant enzymatic activity (39%), whereas mutation to alanine completely abolished activity. In addition, mutation of S911 to alanine altered the migration of hPLD1 by Western analysis (data not shown). In brief, the heterogeneous band observed in Figure 2 can be resolved into a major species and two minor bands of larger apparent molecular weight. The relative ratio of the major and minor bands is not altered by dephosphorylation using alkaline phosphatase, indicating that the heterogeneity does not arise from phosphorylation (in contrast, yeast PLD is phosphorylated and undergoes a shift in mobility after phosphatase treatment; A.Rudge and

Fig. 5. Mutation of conserved amino acids in PLD1. 25 site-directed mutants of PLD1 were assayed for activity in the presence of ARF1. Activities relative to wild-type PLD1 and the control plasmid (Gbx-2) are shown. Two mutants (Y778T and Y778F) were unstable as determined by Western blot analysis and are not shown here. Mutants K898R and G910A-S911T interacted with RhoA-V14. Mutants at aa778 and D903E did not. The experiment was repeated several times. An average of two or more determinations is shown. CR; conserved region.

Table I. PLD activities^a of individual hPLD1 mutants

Mutants	Basal	PLD activators			
		ARF	PKCα	Rac1	RhoA
CR Ib					
L405D	0 ± 5	0 ± 1	0 ± 2	0 ± 1	0 ± 2
G412A	80 ± 8	85 ± 5	77 ± 18	59 ± 10	80 ± 7
G412E	64 ± 23	88 ± 4	95 ± 8	70 ± 12	83 ± 31
CR II					
H464D	3 ± 3	0 ± 1	0 ± 2	0 ± 1	0 ± 1
H464D-K466R	2 ± 11	0 ± 2	1 ± 4	0 ± 1	0 ± 2
H464G	21 ± 14	0 ± 1	0 ± 2	3 ± 6	1 ± 4
H464G-K466G	7 ± 13	0 ± 1	2 ± 3	3 ± 4	0 ± 1
H464G-K466R	32 ± 12	1 ± 1	1 ± 2	3 ± 4	1 ± 1
G478A-G479A	5 ± 10	21 ± 16	17 ± 10	11 ± 8	7 ± 5
G478V	15 ± 2	17 ± 2	11 ± 9	8 ± 9	9 ± 10
G479A	23 ± 22	1 ± 1	0 ± 1	0 ± 1	0 ± 1
G479V	0 ± 7	0 ± 1	0 ± 1	0 ± 2	0 ± 2
CR III					
Y778C	47 ± 6	40 ± 3	49 ± 7	29 ± 7	28 ± 7
Y778G	0 ± 6	0 ± 1	0 ± 1	0 ± 1	1 ± 2
Y778I	62 ± 35	45 ± 1	56 ± 5	28 ± 3	32 ± 8
Y778S	0 ± 25	14 ± 1	10 ± 6	11 ± 2	11 ± 2
CR IV					
K898R	0 ± 2	1 ± 1	0 ± 1	1 ± 3	0 ± 2
D903E	0 ± 7	5 ± 3	3 ± 1	2 ± 1	4 ± 3
G910A-S911T	0 ± 8	13 ± 1	7 ± 1	6 ± 4	11 ± 2

Data represent the mean SD of at least three independent experiments, each carried out in duplicate.

^aExpressed as a percentage of PLD activity with respect to PLD WT (100%, positive control) and Gbx2 (0%, negative control).

J.Engebrecht, submitted). The larger molecular weight bands are also unaltered by mutations to the HKD motif, but are reduced by mutation of S911 to threonine and eliminated by mutation of S911 to alanine (data not shown). One possibility raised by these results is that these larger bands represent catalytic intermediates and that a critical step in catalysis involves modification of S911 by transphosphatidylation (see Discussion).

IYIENQFF motif. This motif is found only in eukaryotic

PLDs, although computer analysis suggests that a region with significant similarity, highlighted by an invariant amino acid (F784), is present in CLS and PSS as well. A possible role for this site is suggested by studies of acetylcholinesterase, which have shown that choline interacts with the enzyme via an aromatic region dominated by phenylalanine and tyrosines (Harel *et al.*, 1993; Gilson *et al.*, 1994). The interaction stabilizes the receptor-substrate complex while catalysis takes place elsewhere. Thus, this aromatic-rich region in PLD represents a

possible binding site for the choline portion of PC. We targeted one of the most conserved amino acids with a series of substitutions (Y777→F, T, S, C, I and G) designed to: (i) keep the aromaticity but remove the hydroxyl group (F); (ii) remove the aromaticity but keep the hydroxyl group (T and S); (iii) remove the aromaticity and the hydroxyl group but keep the side group polar (C); (iv) remove the aromaticity and the hydroxyl group but keep the size of the side group similar (I) or (v) minimize the size of the small group (G). Since two of the six mutant proteins were unstable (T and F) and since the others did not interact with RhoA, this would appear to represent a sensitive site for PLD protein structure. The loss of the aromatic part of the side chain (S, I and C) correlated with a decrease in PLD activity regardless of whether the side chain was polar or not. Substitution of glycine for the tyrosine resulted in a complete loss of activity, suggesting that additional structural constraints were introduced. Taken together, the results suggest that this conserved site plays an important role in enzymatic activity, although it is not as critical as the HKD motif.

CRIb. This region is conserved in all PLD homologs and we targeted two invariant amino acids. A non-conservative substitution at L405 resulted in complete loss of PLD activity. However, semi-conservative (G412A) or non-conserved (G412E) substitutions at the other site had no effect. This result was surprising since the glycine residue at aa412 is completely invariant and presumably is important for some aspect of PLD function common to all the homologs. This finding prompted us to investigate whether the PLD mutants were functional in a biological context, using yeast.

Functional analysis of PLD mutants in yeast

Several hypothetical roles have been proposed for mammalian PLD in a cellular context (Frohman and Morris, 1996). PLD's role in yeast is better understood, since it was identified during a genetic screen as being required for meiosis (Rose *et al.*, 1995). Yeast PLD (SPO14) is a large protein (1683 aa) compared with other PLD cognates. For example, human PLDs range in size from 932 to 1074 aa, castor bean PLD encodes 811 aa and *Streptomyces* PLD, 556 aa. It is not known what function(s) are mediated by the non-conserved sequences in SPO14, although analyses of yeast expressing only the (non-catalytic, non-conserved) amino-terminus suggested that this region interacts with unknown cellular components (Rose *et al.*, 1995). When expressed in yeast lacking SPO14, the amino-terminal region induced a meiotic phenotype more severe than the one which was observed when the entire protein was absent. Moreover, the amino-terminal region exhibited dominant negative effects on meiosis when expressed in wild-type yeast, confirming that this region functions in some manner other than to promote catalysis by the intact protein. These results suggest that the meiotic block observed in yeast lacking SPO14 might be caused by insufficiencies other than a lack of hydrolysis of PC to form PA. To test this hypothesis, site-directed mutagenesis of SPO14 and functional analyses were carried out.

Starvation conditions trigger yeast to undergo meiosis. The most visible manifestation occurs during late meiosis, when the meiotic products become encapsulated into

spores. Tetrads (groups of four spores linked together) are observed most frequently (Figure 6A). In yeast lacking PLD, meiosis is blocked during chromosomal segregation and spores do not form (Figure 6B). The meiotic phenotype can be rescued by transforming the mutant yeast with a centromere-containing plasmid bearing a fragment of genomic DNA containing the *SPO14* gene and flanking DNA (Figure 6C).

To examine the functionality of mutant PLDs in yeast, we generated three point mutants in the SPO14 rescue plasmid and transformed them into yeast lacking SPO14. Levels of PLD activity were determined for the transformed yeast, and their progression through meiosis was examined. Analogous to the *in vitro* analysis of the mutant mammalian PLDs, PLD activity was not observed above background levels for mutants in which substitutions were made in the HKD domain (Table II). Moreover, such mutations eliminated the ability of the plasmid to rescue the meiotic defect (Figure 6D). This result demonstrates that the basis for the sporulation phenotype derives from the loss of PLD catalysis. To address protein stability, Western analysis was performed using an anti-SPO14 antiserum on extracts from yeast expressing the K1098R mutant. The mutant protein was present at levels comparable with that observed for yeast rescued by the wild-type plasmid (data not shown).

In contrast, the G744E mutation (analogous to the G412E mutation made in hPLD1) exhibited full PLD activity (Table II) and rescued the meiotic phenotype equally as well as wild-type SPO14 (Figure 6C). Taken together, the results are paradoxical. On the one hand, G412 is invariantly conserved in all PLD enzymes. On the other hand, it appears to be completely dispensable for PLD function, at least when tested in both yeast and mammalian systems. Several reviews of PLD homologies have discussed the conserved amino acids mutated here in detail (Koonin, 1996; Morris *et al.*, 1996; Ponting and Kerr, 1996). Potential functions for genes that exhibit very limited homology to the PLDs have also been discussed, including an endonuclease and several viral proteins. Our results emphasize the importance of testing conserved relationships through experimental analyses.

Requirement for the HKD motif in a vaccinia viral protein with weak sequence homology to PLD

Two genes encoding proteins with limited homology to PLD are present in vaccinia virus and other poxviruses (Figure 1). K4 contains two HKD motifs and adjacent conserved sequences, while VP37 contains only one partially conserved motif (NxKxxxxD), denoted here as (H)KD. Functionally, however, VP37 appears more important than K4. Vaccinia virus mutants lacking K4 replicate and spread as efficiently as the wild-type virus, suggesting that K4 is redundant, at least in the laboratory setting (Blasco and Moss, 1991). In contrast, viral mutants lacking the gene encoding VP37 (F13L) are seriously compromised (Blasco and Moss, 1991). In cells infected by the wild-type virus, viral particles become enwrapped sequentially in a double layer of membranes derived from the intermediate compartment between the endoplasmic reticulum and *cis*-Golgi, and then a double layer of modified *trans*-Golgi membranes, before leaving the cells (Ichihashi *et al.*, 1971; Sodeik *et al.*, 1993; Schmelz *et al.*,

Fig. 6. The HKD motif is critical for yeast PLD function. Completion of meiosis is marked by the formation of tetrads. (A) Wild-type yeast achieve this at high frequency when stimulated to undergo meiosis. (B) Yeast lacking SPO14 (PLD) do not form tetrads. (C) Yeast transformed with a genomic fragment containing SPO14 and its promoter, or the same fragment engineered to encode the G744E mutation, efficiently rescue the meiotic phenotype. The results were indistinguishable for the wild-type and G744E mutated plasmid. Actually shown is the rescue by the G744E mutated SPO14 gene. (D) Yeast transformed with a genomic fragment containing SPO14 engineered to encode the mutations in the HKD domain do not rescue the meiotic phenotype. The results were indistinguishable for both mutations. Actually shown is the rescue attempt by the SPO14 gene encoding the K1098R mutation.

1994; Strauss, 1995). In cells infected by viral mutants lacking VP37, the *trans*-Golgi envelopment step does not occur (Blasco and Moss, 1991). As a result, viral particle egress is blocked and spread of the virus to adjacent cells greatly diminished. This is visualized as an inability of the mutant virus to form lytic plaques after infection of monolayers of BS-C-1 cells (Blasco and Moss, 1991, 1995).

VP37 localizes to the *trans*-Golgi (Hiller and Weber, 1985; Schmelz *et al.*, 1994) and is retained in the viral membranes (Hirt *et al.*, 1986; Schmutz *et al.*, 1995). hPLD1 is also present in the Golgi apparatus (Colley *et al.*, 1997). hPLD1 has been proposed to regulate budding of vesicles (Stutchfield and Cockcroft, 1993; Chen and Shields, 1996; Frohman and Morris, 1996; Ktistakis *et al.*, 1996; Seethaler *et al.*, 1996; Simon *et al.*, 1996) by generating PA which may act to recruit coatamer proteins (Ktistakis *et al.*, 1996) or cause local alterations in membrane structure (reviewed in Bednarek *et al.*, 1996; Seaman, 1996). Although the sequence homology between VP37 and hPLD1 is quite limited, the additional parallels

Table II. Activity of SPO14⁻ yeast rescued with wild-type and mutant forms of SPO14

Strain	Rescue plasmid	^a PLD activity	^b Spores (%)
SPO14 ⁻	SPO14	1.00	62
SPO14 ⁻	vector alone	0.18	0
SPO14 ⁻	SPO14(K1098R)	0.20	0
SPO14 ⁻	SPO14(G744E)	1.00	59

^aPLD activity was determined as previously described (Rose *et al.*, 1995). Densitometry scanning was performed and normalized for protein content. Activity from the wild-type extract was given a relative value of 1.0.

^bCultures were examined by phase microscopy after 48 h in sporulation medium. The % sporulation was estimated by dividing the number of asci containing two, three or four spores by the total number of cells. At least 300 cells were examined for each strain.

in the subcellular localization and biological roles proposed for the proteins raises two intriguing issues. First, VP37 functionality might depend on the integrity of the (H)KD motif. Second, VP37-mediated viral envelopment might

Fig. 7. Efficient vaccinia virus cell-to-cell spread requires expression of VP37 encoding an intact (H)KD motif. BS-C-1 cells grown in six-well plates were infected with wild-type virus (VP37 WT) or viruses mutated in the (H)KD motif at aa 314 (K→R) or 319 (D→E). After 48 h, cells were fixed and recombinant viruses stained blue by the addition of X-gal. Single wells of a six-well plate are observed in the top row, each well containing at least 15 blue staining plaques. Boxes indicate regions containing infected plaques or cells equivalent to those shown magnified in the bottom row. All magnifications are equivalent. Arrows point to the blue-staining cells infected by mutant viruses.

constitute a novel model for the study of PLD-regulated vesicular budding.

To test the first hypothesis, recombinant vaccinia viruses encoding normal or mutated VP37 proteins were constructed (K314R and D319E; see Figure 1). The viruses additionally carried the *LacZ* gene as a marker. BS-C-1 cell monolayers were infected, and then fixed and stained with X-gal after 48 h of culture. Figure 7 shows the wild-type and mutant plaque sizes. The top panel shows individual wells from a six-well plate, and the bottom panel shows micrographs of wild-type and mutant plaques at equivalent magnifications. Both point mutations in the (H)KD motif drastically diminished the ability of the viruses to form plaques. In fact, cells infected with the mutant viruses could be detected only microscopically, because of the poor virus spread.

Mutant vaccinia virus lacking the F13L gene (VP37) forms very small plaques (Blasco and Moss, 1995) which are indistinguishable from those observed for the (H)KD mutant viruses in Figure 7. To rule out the possibility that the (H)KD point mutations reduced VP37 stability, Western blots were prepared from wild-type and mutant virus infected cells. Unlike the experiment shown in Figure 7 for which limiting quantities of virus were used for infection, in this experiment sufficient virus was added to infect all the cells at the beginning of the culture period. Consequently, the amount of VP37 produced did not depend on subsequent viral spread. For both (H)KD

mutants, VP37 was made in substantial quantity, approximating wild-type levels (data not shown).

Although VP37 is further diverged from PLD than CLS or PSS, these results raised the possibility that VP37 encodes PLD activity. This possibility was addressed using several approaches. First, recombinant VP37 was successfully expressed in COS-7 cells using the expression employed for PLD1. Second, cell extracts prepared from monolayers of BS-C-1 cells infected with wild type or mutant VV were assayed for PLD activity in the presence or absence of ARF1. PLD activity was not observed above background levels in any experiment, suggesting that VP37 does not encode PLD activity (data not shown). It should be noted, however, that the *in vitro* assay is optimized for human PLD1 and may fail to support VP37 activity (for example, yeast PLD is inactive in the mammalian assay system; see Rose *et al.*, 1995). In addition, VP37 expressed alone as a recombinant protein does not accumulate in the *trans*-Golgi network, suggesting that the presence of other viral proteins is required for its correct localization and potentially for its activity (Payne, 1992; Katz *et al.*, 1997).

Finally, the (H)KD motif in VP37 is actually encoded as NKD. We used site-directed mutagenesis to change the VP37 sequence to HKD, but this did not result in VP37 exhibiting PLD activity (data not shown). In addition, we mutated the second HKD motif of PLD1 to NKD, which resulted in a complete loss of activity (Figure 5), suggesting that His and Asn are not readily interchangeable.

Fig. 8. PLD, cardiolipin synthase and phosphatidylserine synthase catalytic reactions. Each reaction involves nucleophile attack on a phosphate bond as shown. Two biochemical activities for PLD have been observed: phosphodiester bond hydrolysis and phospholipid transphosphatidylation. Using H_2O as an electron donor, PLD functions as a phospholipid hydrolysis enzyme, cleaving phosphatidylcholine (PC) to produce phosphatidic acid (PA) and choline. Through the same mechanism but using alcohol instead of H_2O as the electron donor, PLD can generate phosphatidylalcohols. Nucleophiles that can be used by hPLD1 include exogenously supplied butanol or endogenous diacylglycerol (DAG), yielding phosphatidylbutanol (PBut) and bisphosphatidic acid, respectively (Figure 3 and data not shown). CLS and PSS only carry out synthetic reactions in which alcohols are used as the nucleophile, as shown.

Discussion

The PLD superfamily

The identification of non-PLD enzymes (PSS and CLS) as part of the PLD superfamily is intriguing, given PLD's capacity for transphosphatidylation (Figures 3 and 8A). PLD in fact greatly prefers alcohol over water as a nucleophile, and it has never been understood why the endogenous compound (water) is used less efficiently by PLD. Comparisons of the reactions mediated by CLS and PSS are illuminating in this respect (Figure 8B and C). Both enzymes carry out PLD-like reactions, but use substrates containing alcohols as the nucleophile. This relationship suggests that PLD evolved from an ancestral enzyme that was an alcohol transphosphatase and raises the possibility that PLD catalyzes reactions *in vivo* that require transphosphatidylation capacity. Several reports have suggested that PLD may have a role in phospholipid synthesis (Stanacev and Stuhne-Sekalec, 1970; Stanacev *et al.*, 1973; van Blitterswijk and Hilkmann, 1993), and we have observed hPLD1-mediated synthesis of an intriguing phospholipid *in vivo* (below).

A model for catalysis

hPLD has been studied intensively because it is involved in the general signal transduction cascade initiated by a

wide variety of agonists. Members of the ARF, RhoA and PKC families activate hPLD1 in the absence of other protein cofactors, suggesting direct protein-protein contacts (Hammond *et al.*, 1997). None of the mutants created in this study selectively affect the ability of these effectors to activate PLD1, suggesting that the effector interacting sites are located separately from the motifs required for catalysis. This finding was not unexpected, since mammalian PLD2, yeast PLD, plant PLD and prokaryote PLD are all active in the absence of effectors (Morris *et al.*, 1996; Colley *et al.*, 1997).

Our finding that both HKD domains need to be intact for PLD to exhibit catalytic activity implies that the structure mediating catalysis contains both motifs. Although regions II and IV both encode HKD amino acid residues, other highly conserved and requisite amino acids (i.e. S911) are unique to each region (see Figure 1). This suggests that the individual regions mediate analogous but not identical reactions, prompting the following model (Figure 9).

PLD enzymes have two substrates: phosphatidylcholine and water. The PLD reaction could proceed via a ternary complex (i.e. wherein PC and water are both bound to the enzyme at the same site with direct hydrolysis of the phosphodiester bond) or by a substituted enzyme mechanism. The latter would involve the formation of a

Fig. 9. A PLD catalytic model (see text for additional details). At the beginning of the reaction cycle, the amino-, but not the carboxy-terminal HKD histidine is protonated. The first half-reaction involves the formation of a PA–PLD intermediate by covalent linkage of PA to S911. This is effected by donation of S911's proton to the carboxy-terminal HKD histidine, and liberation of a proton from the amino-terminal HKD histidine to form choline. The second half-reaction then reverses this process, as a proton is transferred from a water molecule to the amino-terminal HKD histidine, freeing up the resulting hydroxyl group to hydrolyze the intermediate to form PA. Finally, the ionized serine recovers its proton from the carboxy-terminal HKD histidine.

covalent 'phosphatidylated' intermediate (i.e. PLD–PA) in a half-reaction that would concurrently generate choline. The intermediate would then be hydrolyzed by water, generating free phosphatidic acid. Although the PLD reaction mechanism has been speculated to involve such an intermediate (Stanacev and Stuhne-Sekalec, 1970), no direct evidence has been presented thus far to confirm it. These types of intermediate generally involve the formation of a phosphodiester bond that couples the reaction product to one of the enzyme's serine or threonine residues. Such an essential serine or threonine catalytic residue would be expected to be highly conserved among members of the PLD family, the most obvious choice for which is S911. All members of the superfamily encode serine or threonine at aa911 except PSS, which encodes threonine at the adjacent position (Figure 1).

This two-step mechanism offers an attractive explanation for the apparent 'duplication' of essential catalytic amino acid residues. Histidine residues are frequently involved in activation of nucleophiles in hydrolysis reactions, as has been demonstrated for RNaseA (reviewed in Fersht, 1985). Intriguingly, a bacterial nuclease gene (*nuc*) exists which is an extremely divergent member of the PLD superfamily (Ponting and Kerr, 1996). We propose that catalysis will proceed as follows: (i) activation of an enzyme-intrinsic nucleophile (S911) by transfer of a proton to the carboxy-terminal HKD motif, which promotes transphosphatidylation of S911 with concomitant generation of choline using a proton donated by the amino-terminal HKD motif and (ii) activation of water as a nucleophile by transfer of a proton to the amino-terminal HKD motif, promoting the release of PA via hydrolysis of the intermediate through the donation of a proton from the carboxy-terminal HKD motif. Our findings that the presence of a serine or threonine at position 911 is critical for enzyme activity and that a potential reaction intermediate observed for wild-type PLD1 fails to be observed for hPLD1(S911A) support this model. The Lys and Asp amino acids may play critical roles in stabilizing the His and Ser residues, respectively.

Point mutants resulting in a defect in ability of vaccinia virus to spread

Despite the fact that the vaccinia virus VP37 protein contains only one partially conserved HKD domain, it appears to require this site to be functional. This implies that VP37 either binds to phospholipids or carries out a modifying activity. The former represents a more likely possibility upon first inspection, since all other PLD homologs exhibiting catalysis encode two HKD motifs. However, VP37 can be immunoprecipitated as a dimer from infected cells (Schmutz *et al.*, 1995), suggesting that it may exist functionally in a form in which two intermolecular motifs are brought together. It does not appear likely that VP37 encodes PLD activity. We have also successfully expressed K4, but it does not exhibit PLD activity under our *in vitro* or *in vivo* assay conditions (data not shown).

Since VP37 is considerably further diverged from PLD than CLS or PSS, it is not unlikely to generate or bind phospholipid products other than PA. One possibility for its function may involve an unusual but related phospholipid, semilyso bisphosphatidic acid (SLBPA). Bisphosphatidic acid (BPA) is a phospholipid formed through transphosphatidylation when diacylglycerol is used as a nucleophile donor instead of water (van Blitterswijk and Hilkmann, 1993). The resulting product consequently contains a head group linked to four fatty acid side chains. SLBPA is potentially formed either by PLA2-mediated hydrolysis of BPA, by the conjugation of lysoDAG to PC or by the conjugation of DAG to lysoPC, all of which would result in the formation of a product consisting of a head group linked to three fatty acid side chains. This unusual lipid should promote membrane curvature (van Blitterswijk and Hilkmann, 1993) and thus might assist in the formation of small organelles such as encapsulated viral particles. SLBPA is a normal component of Golgi membranes at low levels (Cluett and Machamer, 1996), but is a major component of the outer envelope of vaccinia virus (Sodeik *et al.*, 1993). Thus, VP37 might mediate concentration of SLBPA into viral membranes. The alternative hypothesis, that VP37 might synthesize

SLBPA, is less attractive because levels of SLBPA are similar in infected and uninfected cells (Cluett and Machamer, 1996).

Curiously, PLD itself has been proposed to synthesize BPA (van Blitterswijk and Hilkmann, 1993). We have examined this by expressing hPLD1 and mPLD2 *in vivo*. Overexpression of hPLD1 in COS-7 cells leads to accumulation of BPA and possibly SLBPA, whereas overexpression of mPLD2 does not (data not shown). The results suggest that the catalytic selectivities of PLD1 and PLD2 may be subtly different, or may reflect the availability of the appropriate substrates in the Golgi and in the plasma membrane, where PLD1 and PLD2 localize, respectively. Nonetheless, this leads to the hypothesis that PLD1 and VP37 may work together to promote viral egress, and suggests that agents which target either activity may represent novel approaches to inhibiting poxvirus pathogenicity.

Materials and methods

General reagents

All phospholipids were purchased from Avanti polar lipids. PIP₂ was isolated as described (Hammond *et al.*, 1995). L- α -dipalmitoyl phosphatidylcholine [choline-methyl-³H] ([³H]PC) was obtained from American Radiolabeled Chemicals and [³²P]phosphoric acid from ICN pharmaceuticals. All other reagents were obtained from previously noted standard sources and were of analytical grade unless otherwise specified (Hammond *et al.*, 1995).

Site-directed mutagenesis

Site-directed mutagenesis of expression plasmids was carried out using the Quik-change kit (Stratagene). Plasmids were sequenced to confirm the intended mutation and the integrity of the surrounding sequences for at least 100 bp using Sequenase (US Biochemicals).

Cell culture

COS-7 cells were maintained in DMEM with 10% FCS. For transfections, the cells were grown in 35 mm dishes and then switched into Opti-MEM I media (Gibco BRL). For *in vivo* assays, the cells were washed into phosphoric acid-free DMEM media after transfection and labeled with 5 μ Ci of [³²P]phosphoric acid (P_i) per dish for 18 h (van Blitterswijk and Hilkmann, 1993).

PLD assays

Recombinant ARF, RhoA, Rac-1 and PKC α were purified and activated using GTP γ S or PMA as previously described (Hammond *et al.*, 1997). Mammalian PLD activity was measured using the *in vitro* head group release assay and the *in vivo* transphosphatidylation assay as previously described (Wakelam *et al.*, 1991; Brown *et al.*, 1993; Hammond *et al.*, 1995, 1997; Colley *et al.*, 1997). PLD cDNAs were transiently overexpressed in COS-7 cells as previously described using the mammalian expression vector pCGN (Hammond *et al.*, 1995, 1997; Colley *et al.*, 1997). The transfection efficiency was observed to be ~5–20%.

Two-hybrid analysis of PLD1-RhoA interactions

Details will be published elsewhere (Y.Zhang and M.A.Frohman, in preparation). In brief, a carboxy-terminal fragment of wild-type or mutant PLD1 (674–1074 amino acids) was cloned into pBTM116 (Bartel *et al.*, 1993) as a fusion protein with the LexA DNA binding domain. In addition, the entire open reading frame of wild-type RhoA or a dominant active form of RhoA (RhoA-valine 14) was cloned into pAct as a fusion protein with the activation domain of herpes virus protein 16 (VP16). The plasmids were used to transform yeast of opposite mating types, and mated to each other or to yeast containing control plasmids. The resulting hybrid strains were expanded in liquid culture and assessed for β -gal activity.

Yeast strains and plasmids

Yeast media were prepared and genetic methods carried out using standard protocols (Rose *et al.*, 1990). The *SPO14* complementing genomic fragment was subcloned into the *LEU2 CEN* plasmid UN105

(Elledge and Davis, 1988). Plasmids were introduced into the diploid yeast strain KR52-3C (Rose *et al.*, 1995), which is homozygous for a *SPO14* deletion, using the lithium acetate transformation procedure (Ito *et al.*, 1983). To assess rescue of the meiotic phenotype, the transformed yeast were grown and sporulated as described previously (Rose *et al.*, 1990). Cultures were examined by phase microscopy after 48 h in sporulation medium. PLD assays were performed as previously described (Rose *et al.*, 1990).

Construction and purification of recombinant virus

Plasmid pRB20lacZ, containing the *LacZ* gene inserted between the F13L gene and its downstream flanking sequence, was a gift of R.Blasco. F13L encodes the 37 kDa Viral Protein (VP37). Recombinant viruses expressing mutant F13L genes were derived from a recombinant vaccinia virus, vRB12, whose F13 L gene was replaced by guanine phosphoribosyltransferase (gpt) (Blasco and Moss, 1995). BS-C-1 cells in six-well plates were infected at a multiplicity of 2 p.f.u. per cell. At 3 h after infection, pRB20lacZ or derivatives containing mutated copies of the F13L gene were transfected with DOTAP (Boehringer Mannheim Biochemicals). The cells were harvested 36 h after transfection, frozen and thawed three times, sonicated and then used to infect fresh BS-C-1 monolayers. After 2 h, the liquid medium was replaced with semi-solid medium containing 1% low melting temperature agarose. After 2 days of viral growth, semi-solid medium containing 1 mg/ml 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) (Promega, Madison, WI) was added.

Recombinant viruses staining blue were picked with a Pasteur pipette and resuspended in 0.5 ml of medium, frozen and thawed three times, sonicated and used for a subsequent round of infection. Virus isolates were passaged and picked three times and then amplified on BS-C-1 cells. Viruses from infected cells were analyzed by PCR. All viruses isolated in this manner and analyzed by PCR retained the *gpt* sequence of the vRB12 virus, probably as the result of single crossover recombination. Therefore, the viruses were passed twice through STO cells in the presence of 6-thioguanine to select against virus containing the *gpt* gene (Isaacs *et al.*, 1990). Blue plaques were picked again under semi-solid medium containing X-gal, and stocks were grown and analyzed by PCR. Pure double crossover recombinant viruses were used in subsequent experiments. For photography, infected monolayers were fixed at 48 h post infection and stained with X-gal.

Western blotting and PLD assays

Cells in six-well plates were infected with 10 p.f.u. of virus per cell. After 27 h, the cells were harvested and subsequently boiled in loading buffer containing 2% SDS, 50 mM Tris-HCl, 10% glycerol in the presence of 2% 2-mercaptoethanol and electrophoresed on 4–20% polyacrylamide gels (Integrated Separation Systems). Proteins were transferred to PVDF membrane (Millipore, Bedford, MA) overnight at 80 mA. Membranes were blocked in 0.1% NP-40, 1% powdered milk, 0.14 M NaCl, 25 mM Tris (pH 7.4) for 30 min followed by incubation with anti-VP37 primary rat monoclonal antibody 15B6 (the kind gift of G.Hiller, see Hiller *et al.*, 1981) supernatant for 2 h. Membranes were washed and incubated with a 1:1000 dilution of alkaline phosphatase conjugated anti-rat secondary (Promega, Madison, WI) for at least 45 min. The membranes were washed three times in blocking buffer and incubated with Western Blue (Promega) substrate solution. The reaction was stopped by rinsing in water.

For recombinant PLD activity analyses, VP37 (F13L) and K4 DNA coding regions were subcloned into pCGN, transfected into COS-7 cells, and assayed as described for hPLD1. Intact virus was assayed by infecting monolayers of BS-C-1 cells for 27 h as described above, following which PLD activity was assayed in the presence or absence of ARF as described previously.

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