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Identification of caspase 3 motifs and critical aspartate residues in human Phospholipase D1b and Phopsholipase D2a.

Michelle H Wright, Michelle J Farquhar, Mina-olga Aletrari, Graham Ladds and Matthew N Hodgkin

Department of Biological Sciences, University of Warwick, Coventry West Midlands CV4 7AL United Kingdom

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ABSTRACT

Stimulation of mammalian cells frequently initiates phospholipase D-catalysed hydrolysis of phosphatidylcholine in the plasma membrane to yield phosphatidic acid (PA) a novel lipid messenger. PA plays a regulatory role in important cellular processes such as secretion, cellular shape change and movement. A number of studies have highlighted that PLD-based signalling also plays a pro-mitogenic and pro-survival role in cells and therefore anti-apoptotic. We show that human PLD1b and PLD2a contain functional caspase-3 cleavage sites and identify the critical aspartate residues within PLD1b that affect its activation by phorbol esters and attenuate phosphatidylcholine hydrolysis during apoptosis.

1. INTRODUCTION

In mammalian cells, many agonists stimulate concurrent activation of phospholipase C (PLC), phospholipase A_2 (PLA_2) phosphatidylinositol 3-kinase and phospholipase D (PLD)-catalysed hydrolysis of phosphatidylcholine (PtdCho) to yield the novel lipid messenger phosphatidic acid (PA) [1-3]. In human cells, PLD1b and PLD2a are the most commonly expressed variants; both contain the characteristic HKD catalytic triad (the 'PLD domain') characteristic of members of the PLD-superfamily and have similar domain structures [1,2,4]. The role of ADP-ribosylation factors (Arfs), the Rho-family of proteins, protein kinase C (PKC) and polyphosphoinositides in regulating phospholipase D activity and localisation is now largely understood [1,2, 5-7].

PLD-catalysed PtdCho hydrolysis plays a regulatory role in important processes such as the oxidative burst in neutrophils [8,9], secretion [10-12], mitogenesis [13,14] and cellular shape change and adhesion [15-17].
Changes in the dynamics of the actin cytoskeleton may represent the common target for PLD signalling for at least some of these processes [15]. We have recently shown a functional link between role and regulation of PLD1b through association with F-actin during antigen-induced secretion in RBL-2H3 cells [17].

Apoptosis is a highly regulated pathway for cellular execution, initiated by changes in the balance of pro-mitogenic/differentiation signals versus pro-death signals [3, 18,19] that is characterised by membrane blebbing, phosphatidylserine exposure, cytosolic shrinkage, mitochondrial and nuclear disruption[18,19]. Apoptotic cell death is implemented following auto-activation of a class of cysteine proteases (caspases), which specifically cleave protein targets containing appropriate aspartate residue within consensus motifs [19,20]. Ligation of cell-surface death receptors leads

to recruitment of adaptor proteins and subsequent activation of caspase-8 that converts inactive caspases-3 and -7 to the active forms, which execute the apoptotic program [18-22]. Known caspase substrates include cell adhesion molecules (e.g. Cadherins), cytoskeletal proteins (e.g. actin and gelsolin), nuclear proteins (lamins), cell-cycle proteins (cyclin A/E), DNA metabolising enzymes (PARP), transcription factors (NF-kB) and signal transduction proteins including receptors, adaptors, kinases, phosphatases and PLA_2 [21,22].

In some cells phosphatidylcholine hydrolysis has been reported to increase during apoptosis [23-25], whereas in others PLD activity has been reported to decrease [26-28]. Furthermore, inhibiting PLD activity can initiate apoptosis [29,30] with over-expression of PLD1 or PLD2 protecting various cancerous cells from apoptosis [31-34], possibly via mechanisms that stabilises p53 via activation of mToR [35,36] and/or Raf1 or Ral [33]. Thus PLD-based signalling is most likely to be pro-survival and anti-apoptotic [1-3, 25-35]. It is possible that PLD represents a legitimate target for the apoptotic machinery to enable plasma membrane remodelling [2] and necessary disruption of the cytoskeleton. Recently, PLD1 signalling has been shown to be anti-apoptotic in calphostin-c treated smooth muscle cells and inhibition of PLD activity by calphostin-c promoted inhibition of microtubule reorganisation [37]. Here we show that human PLD1b and PLD2a contain functional caspase-3 cleavage sites and identify the critical aspartate residues within PLD1b that affect its activation by phorbol esters and attenuate phosphatidylcholine hydrolysis during apoptosis.

2. MATERIAL AND METHODS

Bioinformatic analysis of phospholipase sequences.

Sequences for human PLD1 (accession number **Q13393-2**) and PLD2 (accession number **O14939**), rodent phospholipases D, phopholipase C and phospholipase A2 were analysed using the Predictor of Protease Specificity (PoPS) web-based bioinformatics tool from Monash University (http://pops.csse.monash.edu.au/). PoPS provides an arithmetic score for detected sites based on the consensus sequences for the selected protease. Five residues are used by the program to score a caspase-3 site within a sequence; these are the 4 residues N-terminal of the cleaved peptide bond (S1-S4) and the first C-terminal residue (S1'). An ideal caspase site would score 25 and weak sites can be filtered from the report using a selected threshold, which was specified at 15 in this study. Results from the PoPS were compared to motif searching within the phospholipase D sequences using 4 residue caspase sites with the consensus sequence of DXXD or D/E, D/E, X, D [20].

Generation of vectors expressing GFP-PLD mutatants

The open readings frames (ORF) for human PLD1b or PLD2a were cloned in to pcDNA3.1 with an N-terminal GFP tag. Regions from PLD1b or PLD2a were sub-cloned in to pBluescript using convenient restriction sites and specified aspartate codons changed to alanine by site-directed PCR mutagenesis. The sub-cloned fragments were re-inserted in to the appropriate human PLD ORF in pcDNA3.1. The presence of the mutation within the reconstructed ORF was confirmed by sequencing.

Digestion of *in vitro*-produced PLD proteins by Caspase 3

2μg plasmid mini-prep DNA (pcDNA3.1 harbouring GFP-PLD1b/PLD2a or mutants; predicted molecular weights of 148kDa and 134kDa respectively) was incubated with coupled *in vitro* transcription and translation reagent (Promega) and 100μCi [^{35}]S-methionine (90 minutes at 30^{o}C). Produced PLD proteins were incubated with 25nM caspase-3 (Biomol) in assay buffer (50mM HEPES pH7.4, 100mM NaCl, 0.1% CHAPS, 1mM EDTA, 10% glycerol, 10mM DTT). The competitive inhibitor Acetyl-DDVD-Cho (Pharmingen) was incubated with caspase for 15 minutes prior to mixing with substrates. Proteins were separated by SDS-PAGE (4-12% Tris-glycine gels) and stained with coomassie blue dye. Dried gels were exposed to a storage phosphor screen (Molecular Probes) for 90 minutes before visualisation and quantification of radioactivity (Total-Lab v1). Radioactivity per band was plotted as a percentage of total radioactivity per lane (units). Recombinant PLD1b was produced as described [6,17], digested with caspase 3 (as above) and fragments identified by western blotting using the specified antibodies.

Cell culture and transfection

HEK-293 cells, grown in Dulbecco's modified eagles medium (DMEM) with pyruvate and glutamax (Invitrogen), plus foetal calf serum (10%v/v), penicillin (100U) and streptomycin (100mg/ml) at 37^{o}C in humidified 5% CO2 and passaged at 90% confluency. Two hours prior to transfection growth media was substituted for DMEM without additions. Transfection complexes were prepared in DMEM (without additions) by complexing 6μg DNA with 6μl Plus™ reagent for 15 minutes followed by 5μl of Lipofectamine (Invitrogen) in a final volume of 100μl and incubated with cells for 4 hours before media was replaced with supplemented DMEM. Where necessary cells were grown on coated cover-slips, washed in phosphate-buffered

saline, fixed in paraformaldehyde and visualised using a Leica con-focal microscope at 488nm.

Measurement of phospholipase D activity

HEK-293 cells, transfected as described, were quiesced and labelled with 2μCi/ml [^{3}H] palmitic acid overnight (0.1%BSA, 0.1M HEPES pH7.4), washed twice with 0.1%BSA, 0.1M HEPES, pH7.4 and incubated with 0.3% butan-1-ol for 15 minutes. Cells were stimulated with phorbol ester (PMA) for 30 minutes and lipids extracted with ice-cold methanol, chloroform and water. The lipid phase was dried under N_2 gas and re-suspended in chloroform prior to separation by thin layer chromatography (TLC) developed in chloroform:methanol:acetone:acetic acid and phosphatidylbutanol (PtdBut) was visualised in iodine vapour scraped and counted (expressed as percentage of total lipid labelling).

3. RESULTS

Searches of the protein sequences of human PLD1b and PLD2a with two consensus motifs for caspase 3 cleavage (DXXD or D/E, D/E, X, D) [20] identified several putative sites. The Predictor of Protease Specificity (PoPS) web-based bioinformatics tool provided quantitative scores for the putative caspase cleavage sites within PLD sequences. Within PoPS, caspase 3 sites are defined using four residues N-terminal (S4-S1) and one residue C-terminal (S1') to the cleaved peptide bond to give a maximum achievable score of 25. PoPS analysis identified 3 putative caspase-3 sites (with scores above 15) in the protein sequence of human PLD1b and 2 putative sites in human PLD2a (Figure 1A). All of these sites had been identified using the consensus sequence approach.

In human PLD1b, two of the putative caspase sites are located in the 'loop region' at $DDVD_{549}S$ and $DFID_{631}R$ between the PLD domains (Figure 1B). The remaining caspase 3 site in the N-terminus of PLD1b at $EEVD_{41}Y$, is known to be the region of PKC interaction and phosphorylation sites (Figure 1B) [17]. Two high-scoring putative caspase-3 sites were also identified in the N-terminus of human PLD2a (Figure 1A) at $DELD_{16}S$ and $DEVD_{28}T$. Of the 3 caspase sites identified in human PLD1b, $DFID_{669}R$ is conserved in the rat, but not the mouse, PLD1 sequence. Of the 2 caspase-3 sites found in human PLD2a, $DEVD_{28}T$ is conserved within the mouse but not rat PLD2 sequences. Analysis of the sequences of phospholipases C (β, γ, δ) and various phospholipase A2 variants demonstrated that the occurrence of these putative caspase 3 sites is specific to phospholipases D (results not shown).

To determine if PLD1b was a caspase-3 substrate, purified recombinant and catalytically active [6,17] GST-PLD1b (1μg) was incubated with purified recombinant caspase-3 (100ng) at 37^{o}C for 0-60 minutes and PLD1b fragments

separated by SDS-PAGE and detected by western blotting with commercial antibodies against an internal PLD1b sequence (Biosource; PLD1b residues 523-540) and the C-terminus (Santa Cruz). Figure 1C shows that human recombinant PLD1b is rapidly (within 10 minutes) cleaved by caspase-3 in to two fragments; one of 75-90kDa (F1) recognised by the internal antibody and one of 50-60kDa (F2) recognised by the C-terminal antibody. The sizes of the produced fragments were consistent with cleavage of GST-hPLD1b at one of the two centrally placed putative caspase-3 sites, rather than D_{41}. PLD1b degradation was inhibited by pre-incubating caspase-3 with the competitive peptide inhibitor Ac-DEVD-Cho [22], but not prevented by inclusion of excess free glutathione S-transferase. The larger PLD1b fragment (75-90kDa) was also identified by antibodies raised against the N-terminal of PLD1b (Biosource) and by an anti-GST (Sigma; results not shown), suggesting that it consisted of at least residues 1-540 of PLD1b and the intact GST tag. GST-hPLD1b remained caspase-3 sensitive at caspase to PLD1b ratios of 1:25 and 1:100 (results not shown).

To identify which aspartate residues were responsible for directing caspase-mediated cleavage of PLD1b, D_{41}, D_{545} and D_{631} were mutated to alanine and proteins expressed by coupled *in vitro* transcription and translation in the presence of ^{35}S-methionine. Labelled PLD1b proteins were incubated with caspase-3 and fragments separated by SDS-PAGE prior to identification and qunatification by phosphorimage analysis. Figure 2A shows that wild type GFP-PLD1b is successfully expressed *in vitro* and is rapidly degraded by caspase-3 to two fragments; one of 75-90kDa (F1) and one of approximately 50kDa (F2). Mutation of D_{545} to alanine renders PLD1b insensitive to caspase-mediated cleavage whereas mutation of D_{631} does not prevent cleavage. Cleavage is specific to caspase-3 since pre-incubation of caspase 3 with the peptide competitive inhibitor (Ac-DEVD-Cho) prevents PLD1b degradation.

Mutation of D_{41} to alanine did not prevent cleavage of PLD1b (results not shown). Figure 2b shows quantification of radioactivity within the 148kDa parent GFP-hPLD1b (wild-type and mutants) band following incubation with caspase-3 from 3 separate experiments. As a control human GFP-hPLD2a (134kDa) was also expressed using *in vitro* coupled transcription and translation. Mutation of D_{16}, but not D_{28}, to alanine renders human PLD2a caspase-3 insensitive (Fig. 2C and 2D). Figure 3A shows that approximately half of the *in vitro* produced GFP-PLD1b protein (at 148kDa) is degraded by caspase-3 within 10 minutes with a concomitant increase in radioactivity in the 70-80kDa fragment (F1). Degradation of GFP-PLD1b is dependent on caspase-3 concentration with near maximal levels of degradation occurring at 15-20nM caspase 3 (Fig. 3B).

GFP, GFP-PLD1b or GFP-PLD1b$_{D545A}$ and GFP-PLD1b$_{D631A}$were successfully expressed by transient transfection of human embryonic kidney cells (HEK-293) as determined by con-focal microscopy. Figure 4A shows that GFP is more weakly expressed than wild type PLD1b or the PLD1b mutants. Phorbol ester-stimulated PLD activation was measured in HEK 293 cells expressing GFP alone. The results in figure 4B show that increasing concentrations of phorbol ester result in a 4-fold increase in PLD activity as determined by formation of phosphatidylbutanol. Transfection of HEK-293 with GFP-PLD1b wt or PLD1b mutants resulted in an approximate 3-fold increase in basal activity that was statistically significant. However, only wild type-GFP-PLD1b and GFP-PLD1b$_{D545A}$ showed phorbol ester sensitive increases in PLD activity following transfection, mutation of D_{631} to alanine rendered this mutant insensitive to subsequent phorbol ester mediated stimulation.

HEK-293 cells expressing wild-type GFP-PLD1b were initiated in to apoptosis by UV-light treatment and phorbol ester-stimulated phosphatidylcholine

hydrolysis was measured over 24 hours. During this period there was a 4-fold increase in caspase activity (results not shown) and at 24 hours the cells showed a significant amount of apoptotic phenotypes including membrane blebbing and nuclear condensation and fragmentation (results not shown). In cells expressing wild-type PLD1b, PMA-stimulated phosphatidylcholine hydrolysis initially declined after UV treatment but was followed by a sustained rise in PtdBut accumulation between 4-12 hours (Fig. 4C). Whilst the initial decline in phosphatidylcholine hydrolysis was also apparent in apoptosing cells expressing hPLD1b$_{D545A}$, much of the second sustained phase of phosphatidylcholine hydrolysis observed between 4 and 12 hours was lost (Fig. 4C). This data suggests that over-expression of a caspase-resistant form of PLD1b reduces the progress of phosphatidylcholine hydrolysis in apoptosing cells.

4. DISCUSSION

Both human PLD1b and PLD2a contain previously unrecognised caspase 3 cleavage sites not present in rat or mouse phospholipases D. In PLD2a, the caspase 3 site is located in the N-terminus at residue D_{16} whereas in hPLD1b, the caspase 3 cleavage site is located in the loop region between the HKD motifs at residue D_{549}. The caspase motif identified in PLD1b ($DEVD_{545}S$) is similar the classical caspase site identified in DNA topioisomerase I (DEVD) cytosolic PLA2 (DELD) and cleavage at this site is inhibited by the known peptide competitor DDVD [22,38,39]. Structure and functional analysis has shown that PLD1b activity can be reconstituted when the ORF is separated (genetically) within the loop region and the two halves co-expressed within cells [40]. This observation has lead to the suggestion that interaction of the HKD motifs in the PLD domains, via folding of the loop region, is necessary for catalytic activity [1,40]. Our data suggests that caspase-mediated cleavage of PLD1b could provide a post-translational mechanism that mimics this observation.

Whilst mutation of D_{549} to alanine in PLD1b did not affect phorbol ester activation of PLD1b in HEK 293 cells, mutation of D_{631} to alanine prevented stimulation of phosphatidylcholine hydrolysis. This data suggests that within the loop region D_{631} plays a role in regulating activation of PLD1b. Since the PKC binding and phosphorylation sites within PLD1b and located in the extreme N-terminus [17], the mechanism by which this mutation prevents phosphatidylcholine hydrolysis is not clear.

Expression of the caspase-3 resistant form of PLD1b reduced the extent of UV-initiated phosphatidylcholine hydrolysis in HEK 293 cells undergoing apoptosis. Apoptosis involves a complex interplay of irreversible changes to the plasma-

membrane, cytoskeleton and nucleus and our data suggest that PLD activity may either be targeted for destruction to facilitate these changes or through caspase-mediated degradation become deregulated and therefore able to play a role in facilitating apoptotic changes.

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LEGENDS

Figure 1. Human PLD is a potential caspase-3 substrate.

(A) Putative caspase-3 cleavage sites in human PLD1b and PLD2a were identified and scored (out of 25) using PoPS. (B) Purified recombinant GST-PLD1b (1μg) was incubated with caspase 3 (100ng) for up to 60 minutes with or without Ac-DEVD-Cho inhibitor (2μM) or excess recombinant GST (xsr-GST - 2.5μg) and fragments separated by SDS-PAGE (4-12%) prior to identification by western blotting with hPLD1 specific antibodies raised to an internal sequence (Biosource) or the C-terminal of PLD1b (Santa Cruz) and ECL. (C) A schematic representation of the human PLD1b sequence showing the positions of PX, PH and PLD domains, putative caspase-3 cleavages sites and antibody epitopes.

Figure 2. Identification of caspase-3 motifs PLD1b and PLD2a

Wild-type and mutated forms of human GFP-PLD1b (A) and GFP-PLD2a (B) were expressed *in vitro* in the presence of ^{35}S-methionine. Produced proteins were incubated at 37^{o}C for 60 minutes with caspase-3 with or without pre-incubation with peptide inhibitor and labelled fragments (F1 and F2 for PLD1b and F3 and F4 for PLD2a) separated by SDS-PAGE prior to visualisation and quantification by phosphorimaging (results for PLD1b-panel C, PLD2a -panel D). Images are representative of 3 experiments and bars charts show mean radioactivity remaining in the parent PLD band (1b/2a) as a % of total, +/- SEM. Luciferase (61kDa) expression was a control for the coupled transcription and translation process.

Figure 3. Caspase 3 catalysed cleavage of PLD1b is both time and dose – dependent.

Wild type human GFP-PLD1b was expressed *in vitro* as described. (A) Produced PLD1b protein was incubated at 37^{o}C for up to 120 minutes with caspase 3 (25nM). (B) Produced PLD1b protein was incubated at 37^{o}C for 120 minutes with caspase 3 (0-50nM). Labelled fragments were separated and quantified as before. Graphs show mean radioactivity remaining in the parent PLD1b band (148kDa) in the 70-90kDa (F1) band as a % of total, +/- SEM from 3 experiments.

Figure 4. Expression of a caspase-3 resistant PLD1b in HEK-293 cells.

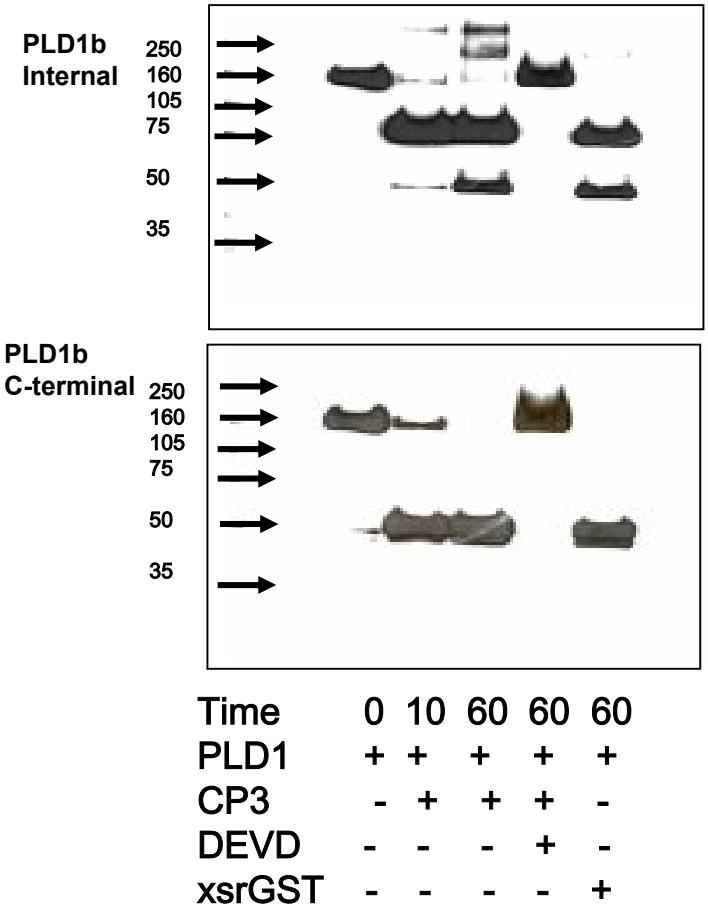
HEK 293 cells transiently expressing GFP, GFP-hPLD1b , $D_{545}A$ or D631A were fixed and GFP-fluorescence localised by con-focal microscopy (panel A). Similarly transfected (GFP, GFP-hPLD1b-wild type, $D_{545}A$ or $D_{631}A$) HEK-293 cells were labelled ^{3}H-palmitate prior to stimulation with phorbol ester (0-300nM) in the presence of butan-1-ol (0.3%w/v). Accumulated phosphatidylbutanol (PtdBut) was separated by thin layer chromatography, extracted and quantified by scintillation counting. Results in panel B are means from triplicate determinations +/- SEM. HEK-293 cells transiently transfected with pcDNA3.1 expressing GFP-hPLD1b or GFP-hPLD1b$D_{545}A$ and labelled with ^{3}H-palmitate prior to initiation in to apoptosis by UV-light treatment. PMA-stimulated (300nM) phosphatidylbutanol accumulation was quantified over 24 hours (panel C) as means from triplicate determinations +/- SEM.

Figure

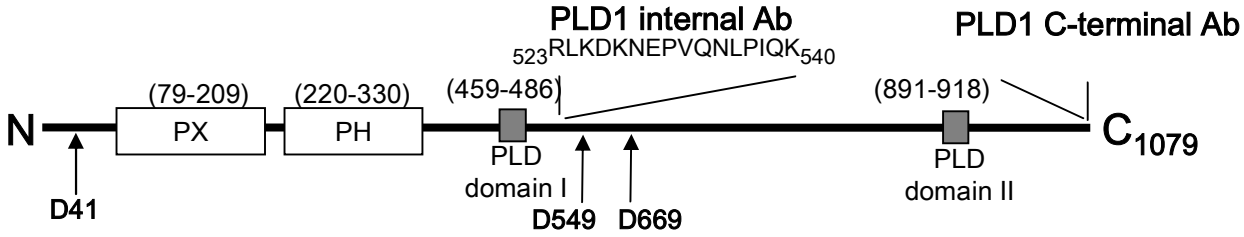
A

hPLD1b Sequence	PoPS Score	hPLD2a sequence	PoPS Score
$EEVD_{41}Y$	16.8	$DELD_{16}S$	20
$DDVD_{549}S$	21.2	$DEVD_{28}T$	22
$DFID_{669}R$	15.3		

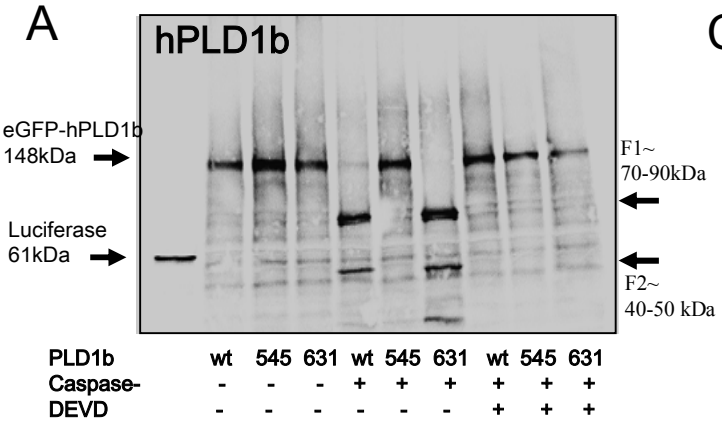
B

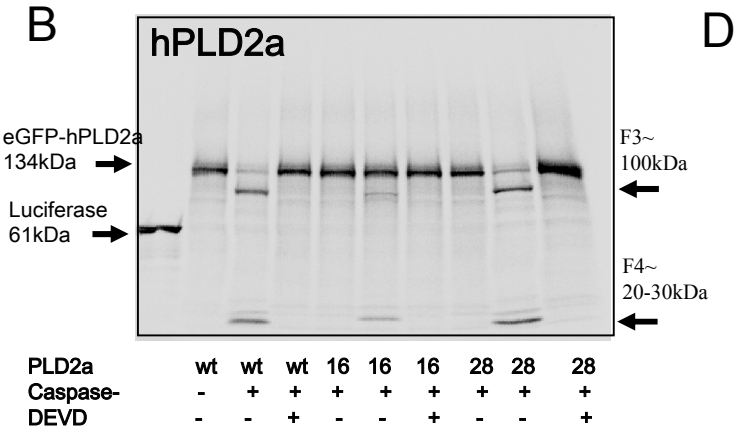


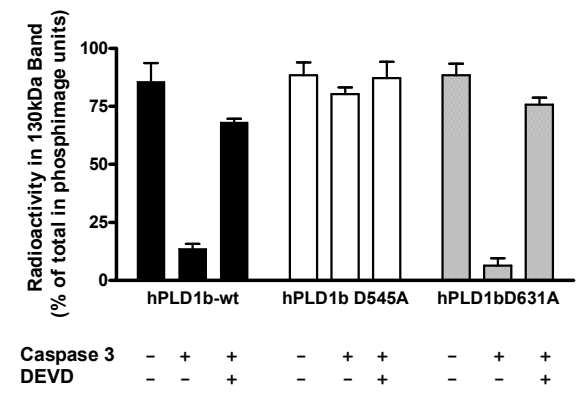
C

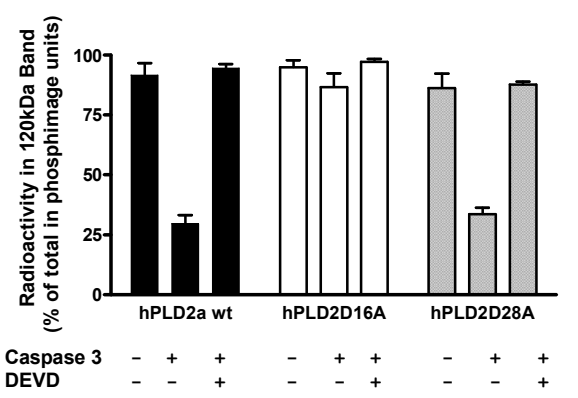


Figure

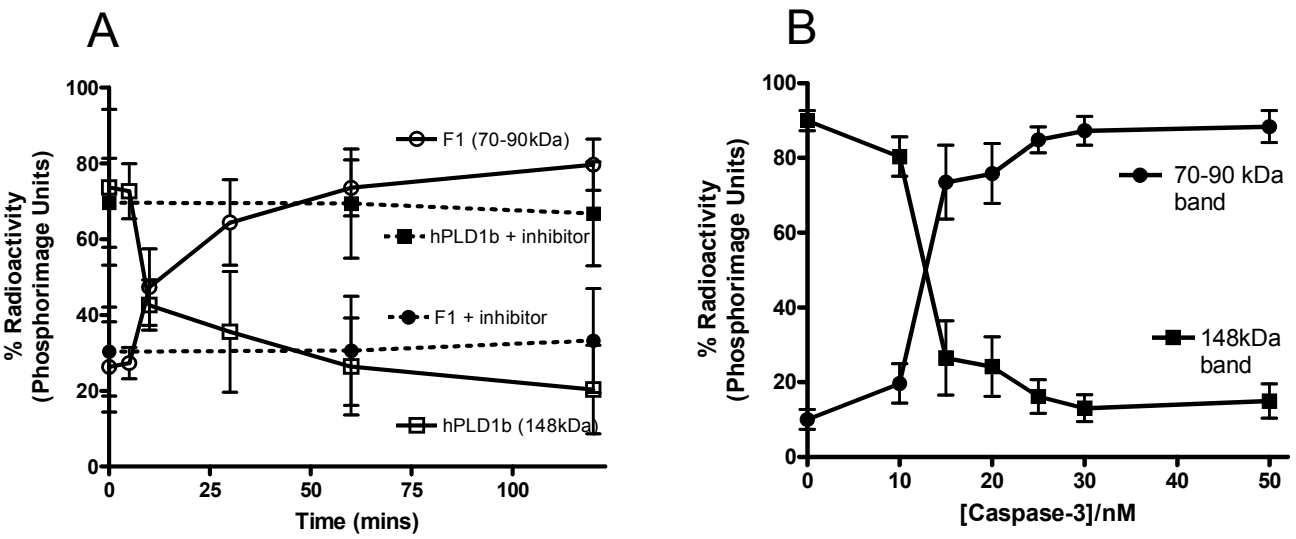

A
hPLD1b
eGFP-hPLD1b
148kDa
Luciferase
61kDa
F1~
70-90kDa
F2~
40-50 kDa
PLD1b wt 545 631 wt 545 631 wt 545 631
Caspase- - - - + + + + + +
DEVD - - - - - - + + +


B
hPLD2a
eGFP-hPLD2a
134kDa
Luciferase
61kDa
F3~
100kDa
F4~
20-30kDa
PLD2a wt wt wt 16 16 16 28 28 28
Caspase- - + + + + + + + +
DEVD - - + - - + - - +

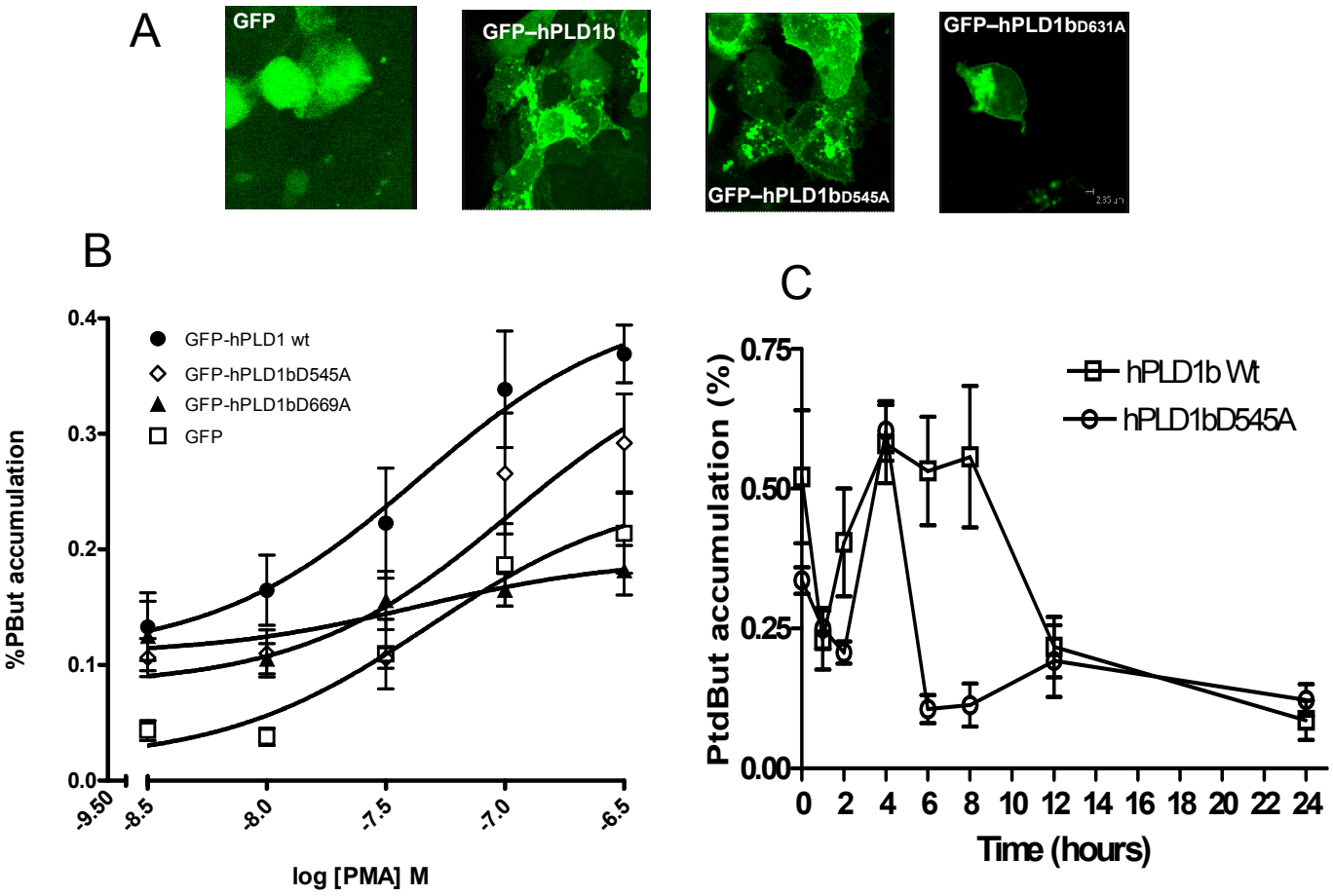

C
Radioactivity in 130kDa Band
(% of total in phosphimage units)
100
75
50
25
0
hPLD1b-wt
hPLD1b D545A
hPLD1bD631A
Caspase 3 - + + - + + - + +
DEVD - - + - - + - - +


D
Radioactivity in 120kDa Band
(% of total in phosphimage units)
100
75
50
25
0
hPLD2a wt
hPLD2D16A
hPLD2D28A
Caspase 3 - + + - + + - + +
DEVD - - + - - + - - +

Figure


A
100
80
60
40
20
0
% Radioactivity
(Phosphorimage Units)
0
25
50
75
100
Time (mins)
F1 (70-90kDa)
hPLD1b + inhibitor
F1 + inhibitor
hPLD1b (148kDa)
B
100
80
60
40
20
0
% Radioactivity
(Phosphorimage Units)
0
10
20
30
40
50
[Caspase-3]/nM
70-90 kDa
band
148kDa
band

Figure


A
GFP
GFP–hPLD1b
GFP–hPLD1bD545A
GFP–hPLD1bD631A
B
GFP-hPLD1 wt
GFP-hPLD1bD545A
GFP-hPLD1bD669A
GFP
%PBut accumulation
log [PMA] M
C
hPLD1b Wt
hPLD1bD545A
PtdBut accumulation (%)
Time (hours)