Endosomal localization of phospholipase D 1a and 1b is defined by the C-termini of the proteins, and is independent of activity

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The factors regulating the activity of cellular phospholipase D (PLD) have been well characterized; however, the cellular distribution of specific PLD isoforms and the factors defining localization are less clear. Two specific PLD1 isoforms, PLD1a and PLD1b, are shown in the present study to be localized in endosomal compartments with early endosomal autoantigen 1, internalizing epidermal growth factor receptor (ErbB1) and lysobisphosphatidic acid. Novel C-terminal splice variants of PLD1, PLD1a2 and PLD1b2, do not exhibit this endosomal localization. Studies using catalytically inactive and C-terminal deletion mutants of the four PLD1 isoforms led to the conclusion that the C-terminus plays an important part in the catalytic activity of PLD1, but that the endosomal localization of PLD1a and PLD1b is defined by the C-terminus and not catalytic activity.

Key words: early endosomal autoantigen 1, endosome, lysobisphosphatidic acid, PLD1.

INTRODUCTION

Phospholipase D (PLD) catalyses the hydrolysis of phosphatidylcholine to phosphatidic acid and choline. In mammalian cells cellular PLD activity is normally low and increases on agonist stimulation of a variety of cell surface receptors [1]. A number of cofactors have been implicated in mediating cellular PLD activation, and studies of the *in vitro* properties of the recently cloned mammalian PLDs have confirmed the potential of these cofactors to regulate PLD1 activity (reviewed in [2–4]). Two separate PLD genes, PLD1 [5,6] and PLD2 [7,8], which are approximately 50 % identical, have been cloned from mammalian cells. PLD1 has been reported to encode splice variants PLD1a and PLD1b [9], the latter of which lacks 38 amino acids from a central region of these otherwise identical proteins. Other cellular PLD activities have been described, including an oleate-activated isoform [10]; however, these remain to be cloned and characterized.

PLD1 and PLD1-like activities have been described in a variety of cytoplasmic and membrane localizations [1,11]. Using subcellular fractionation ADP-ribosylation factor (ARF)-activated PLD has been identified in the plasma membrane [12], secretory granules [13], nuclear membrane [14] and Golgi [15], whereas immuno and direct fluorescence have identified PLD1 in perinuclear vesicles [7,16], secretory vesicles and lysosomes in RBL-2H3 cells [17] and also in late endosomes and lysosomes [18].

The present study identifies the cellular localization of PLD1 isoforms in HeLa cells and, through the use of a novel class of PLD1 splice variant, determines the elements involved in defining catalytic activity and localization of the PLD1 isoforms.

EXPERIMENTAL

Plasmids and constructs

DNA was amplified from DX3 and U937 cell cDNA libraries (pCDM8-DX3, Dr J. Trowsdale, Division of Immunology, Department of Pathology, University of Cambridge, U.K.; pGAD10-U937, Dr L. Dekker, Centre for Molecular Medicine, University College London, U.K.) using primers for the expressed sequence tag (EST)-PLD sequence (accession number R34925; 5′-TCCATGCTAACGTACAGT-3′ and 5′-ATGTCTCCTTCGAACCCT-3′) and for the cDNA library vectors. Amplified fragments were then cloned into the vector pCR-Blunt (Invitrogen) for sequence analysis. DNA was also amplified from DX3 and U937 cell cDNA (extracted with RNeasy, Qiagen; amplified with Superscript, Gibco BRL) using clone-specific oligomers (PLD1a and PLD1b, 5′-GGGGGGGTCGACTTAAGTCCAAACCTCCAT-3′; PLD1a2 and PLD1b2, 5′-GCCCTCGAGTCAGGGATCTTCGACACC-3′) and oligomers designed along the sequence of PLD1a (accession number U38545; 5′-TCTGGCATGGAAAGGAC-3′, 5′-GACTCACTAGACCTCATGCTG-3′, 5′-ACCCATCCAGAAGAGTATTGA-3′, 5′-GTCTACATCCCAACATAAAGGTGA-3′ and 5′-TCCTGCTGGTAGACAAAGAAT-3′) at approximately 400 bp intervals. For protein production PCR-amplified DNA containing the cDNA sequence from the EST (5′-GGAGGATCCCATGCTAACGTACAGTTG-3′) to the C-termini of PLD1a (5′-GGGGGGGTCGACTTAAGTCCAAACCTCCAT-3′) and PLD1a2 (5′-GCCCTCGAGTCAGGGATCTTCGACACC-3′) was cloned into the *Bam*HI and *Xho*I sites of the bacterial expression vector pRSET (His-tag expression vector; Invitrogen) using *Bam*HI and *Sal*I or *Xho*I restriction enzyme sites designed within the oligomers. DNA containing the start of the open reading frame of PLD1 was also PCR-amplified using an oligomer containing a *Bam*HI site (5′-GGGGGGGGATCCATGTCACTGAAAAACGAGCC-3′) and a downstream reverse oligomer (5′-CACAGACTCACAGACGTG-3′). This amplified product was cloned into the *Bgl*II and *Hin*dIII sites of pEGFP-C1 (where GFP is green fluorescent protein; ClonTech Laboratories) and pDsRED1-C1 [encoding red fluorescent protein (RFP); ClonTech Laboratories]. Constructs consisting of full-length PLD1a, PLD1b, PLD1a2, PLD1b2 and deletion

Abbreviations used: AP, adaptor protein; ARF, ADP-ribosylation factor; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EEA1, early endosomal autoantigen 1; EGF-R, epidermal growth factor receptor; EST, expressed sequence tag; GFP, green fluorescent protein; GTP[S], guanosine 5′-[γ-thio]triphosphate; LAMP-1, lysosome-associated membrane protein-1; LBPA, lysobisphosphatidic acid; TGN, *trans*-Golgi network; PLD, phospholipase D; PKC, protein kinase C; RFP, red fluorescent protein.

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mutants PLD1a-976Δ, PLD1b-938Δ, PLD1a-961Δ and PLD1b-923Δ, were generated by cloning the relevant PCR-amplified DNA into the *Hin*dIII and *Sal*I, *Xho*I or *Nhe*I sites of these vectors (976/938, 5′-GCGCCCCGGGGCTAGCTTAATCCT-GAATGTCCTCACTTGGG-3′, containing *Xma*I and *Nhe*I sites; 961/923, 5′-GCGCCCCGGGGCTAGCTTACCTAAA-GCACTGTAGCCGAAGTCC-3′, containing *Xma*I and *Nhe*I sites). Similarly, GFP- and RFP-PLD1 splice variants were generated from a *Bam*HI–*Hin*dIII N-terminal construct in the vectors using the *Hin*dIII, *Xho*I and *Xma*I sites. Constructs containing the catalytic site mutations were engineered using convenient restriction sites from constructs provided by Professor M. J. O. Wakelam (Institute for Cancer Studies, Birmingham University Medical School, Birmingham, U.K.) [17]. Authenticity of all constructs was confirmed by dideoxy sequencing.

Confocal microscopy

HeLa Ohio and HEK-293 cells (ICRF) were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (v/v) foetal bovine serum on acid-washed glass coverslips. Immunocytochemistry was performed 24–48 h after transfection of HeLa cells using Fugene6 (as described in the manufacturer's instructions; Roche) or 24–48 h after transfection of HEK-293 cells transfected as described previously [19]. Cells were either left un-fixed, fixed with 4 % (w/v) paraformaldehyde or fixed and permeabilized for antibody staining before mounting. Cells were mounted, after washing in PBS and H_2O, in mowiol [100 mM Tris/HCl (pH 8.5), 10 % (w/v) mowiol (Calbiochem) and 25 % (v/v) glycerol] with antifade {2.5 % (w/v) 1,4-diazadicyclo[2.2.2]octane; Sigma}. For antibody staining, cells were washed in PBS, permeabilized in PBS/0.2 % Triton X-100 and washed with PBS/1 % (w/v) BSA for blocking. Cells were stained with antibodies to a myc epitope (9E10; ICRF), epidermal growth factor receptor (EGF-R; R1 and F4; ICRF), caveolin, clathrin (Transduction Laboratories), transferrin, early endosomal autoantigen 1 (EEA1), lysosome-associated membrane protein-1 (LAMP1; Santa Cruz Biotechnology), adaptor protein (AP)-1 and AP-2 (Sigma), p115 (ICRF), GM130 (Professor G. Warren, Departmant of Cell Biology, Yale University School of Medicine, New Haven, Connecticut, U.S.A.), cation-independent mannose-6-phosphate receptor (Dr S. A. Tooze, ICRF, London) and lysobisphosphatidic acid (LBPA) [20] with appropriate Cy5- and Cy3-conjugated anti-mouse, -goat and -rabbit antibodies (Jackson Immunoresearch Laboratories). To observe plasma membrane EGF-R, cells were treated with antibodies to EGF-R (R1; ICRF) for 15 min in PBS at 4 °C prior to fixing and mounting as described above. Slides were examined using a confocal laser scanning microscope (Axioplan 2 with LSM 510; Carl Zeiss Inc.) equipped with 63× and 100×/1.4 Plan-APOCHROMAT oil immersion objectives. GFP, Cy3 (and RFP) and Cy5 were excited with the 488 nm, 543 nm and 688 nm lines of Kr–Ar lasers respectively, and individual channels were scanned in series to prevent cross channel bleed through. Each image represents a single approximately 0.8 μm 'Z' optical section of GFP-transfected cells. Quantification of colocalization was carried out by assessing the colours of individual pixels within the image of individual cells extracted from a field using the LSM 5.1 software v2.5. Thresholds were set to eliminate non-vesicular background staining and therefore values represent percentages of predominantly vesicular pixels. Vesicle counting was carried out on randomly selected images of transfected cells from at least three independent experiments and values are given as experimental mean percentages ± S.E.M. for the total number of cells counted (*n*).

Peptide synthesis, immunization and antisera purification

Peptides 1a/b, GTKEAIVPM, and 1a/b2, SKMTPGVEDP, were synthesized and analysed by reverse-phase HPLC and MS by Nicola O'Reilly and Hans Hansen (ICRF, London). Antisera were generated in rabbits from the peptides coupled to keyhole-limpet haemocyanin using glutaraldehyde as described previously [21], and were purified as described previously [22].

Western-blot analysis

For Western-blot analysis protein extracts from approximately 5×10^6 HeLa Ohio cells were prepared in urea sample buffer [8 M urea, 1 % (w/v) SDS, 100 mM Tris (unbuffered), 150 mM NaCl, 50 mM EDTA and 1 % (v/v) 2-mercaptoethanol], and approximately 33 % of this was separated overnight by SDS/PAGE (10 % polyacrylamide). Proteins were transferred 'wet' on to PVDF membranes (Millipore) at 4 °C in 25 mM Tris (unbuffered), 193 mM glycine and 20 % (v/v) methanol buffer for 2 h at 600 mA using a Bio-Rad TransBlot apparatus. Antisera were incubated at 1:500 dilution with blocked [PBS/0.1 % Tween 20/1 % (w/v) milk powder at 25 °C for 1 h] membranes in PBS/0.1 % Tween 20 overnight at 4 °C. Western blots using the anti-GFP monoclonal antibody (3E1; ICRF) were carried out as above except incubation with the primary antibody (1:1000 dilution) was carried out at 25 °C for 1 h. For detection of antigen by chemiluminescence, membranes were washed (in 1 × PBS/0.1 % Tween 20 for 5 min, in 1 × PBS/0.1 % Tween 20/0.5 M NaCl for 5 min, and in 1 × PBS/0.1 % Tween 20 for 5 min) and an appropriate horseradish peroxidase-linked (Amersham) secondary antibody was employed before visualization with ECL® (Amersham). Densitometry was carried out on scanned images using NIH-image (v1.58) software for PowerMacintosh. All cell lines were provided by the ICRF, London.

Fractionation

HEK-293 cells grown in DMEM supplemented with 10 % (v/v) foetal bovine serum were transiently transfected, as previously described [19], with GFP–PLD1a, GFP–PLD1b, GFP–PLD1a2, GFP–PLD1b2, GFP–PLD1a-976Δ, GFP–PLD1b-938Δ, GFP–PLD1a-961Δ and GFP–PLD1b-923Δ. After 48 h cells were washed with cold (4 °C) PBS and resuspended in cold (4 °C) buffer [50 mM Hepes (pH 7.2), 3 mM EGTA, 2.5 mM dithiothreitol (DTT), 3 mM $MgCl_2$, 2 mM $CaCl_2$ and complete protease inhibitors (Roche)] before disruption by homogenization (1 ml glass homogenizer; Fisher Scientific). The extract was then separated by centrifugation at 350000 ***g*** in a TLA100.2 rotor (Beckman) into membrane pellet and supernatant fractions. These fractions were resuspended in 0.5 × urea sample buffer (see above) or an equal volume of 1 × urea sample buffer respectively, and equivalent percentages of each were separated by SDS/PAGE for Western-blot analysis as described above.

PLD assays

HEK-293 cells were maintained and transfected as previously described [19]. PLD activity was assessed *in vivo* by prelabelling cells with 2 μCi/ml [^{3}H]myristic acid (NEN) for 6 h and then adding ethanol to a final concentration of 2 % (v/v) for

15 min (PLD will transphosphatidylate ethanol to produce phosphatidylethanol). Cells were washed and scraped, and lipids were extracted as below for phospholipid analysis. *In vitro* assays were performed using lipid labelled phosphatidylcholine substrate {L-α-dipalmitoyl-[2-palmitoyl-9,10-^{3}H(N)]-; 1 μCi/assay; NEN} presented in a 10 × liposome mix [9 μM dipalmitoyl-phosphatidylcholine (Sigma), 126 μM dipalmitoyl-phosphatidyl-ethanolamine (Sigma) or 12.6 μM dipalmitoyl-phosphatidylinositol 4,5-bisphosphate (Echelon, prepared and quantified as previously described [23]) in 50 mM Hepes (pH 7.2), 3 mM EGTA, 80 mM KCl and 2.5 mM DTT] after bath sonication. This was added to protein extracts prepared in 50 mM Hepes (pH 7.2), 3 mM EGTA, 80 mM KCl, 2.5 mM DTT, 3 mM $MgCl_2$, 2 mM $CaCl_2$, 2% (final) ethanol with or without 50 μM guanosine 5′-[γ-thio]triphosphate (GTP[S]). Assays were terminated by phospholipid extraction using chloroform/methanol/aqueous (8:4:3, by vol.), before separation by TLC, on K6 Silica Gel 60 (Whatman) developed with the organic phase of 2,2,4-trimethylpentane/ethylacetate/acetic acid/H_2O (2:13:3:10, by vol.). Plates were visualized using En^{3}Hance spray (NEN) and samples were scraped for counting in scintillant.

RESULTS AND DISCUSSION

PLD1a and PLD1b are located in endosomes in HeLa cells

PLD1b has been demonstrated to localize to secretory vesicles in rat basophilic leukaemia cells [17], and has been suggested to reside in a variety of cellular localizations in other cell lines (see [1]). To define the cellular localization of PLD1a and PLD1b in HeLa cells, GFP derivatives of PLD1a and PLD1b were constructed and transiently transfected into HeLa cells. Protein analysis by Western blotting using GFP-specific monoclonal antibodies confirmed that full-length GFP–PLD1 isoforms were being produced in these transfected cells (results not shown), and examination by confocal microscopy revealed that GFP–PLD1a and GFP–PLD1b appear predominantly on small perinuclear and cytoplasmic vesicular structures as has been described before [7,18,24]. These observations were confirmed by examination of transfected cells prepared using GFP-specific monoclonal antibodies and Cy5-conjugated anti-mouse secondary antibodies and also with RFP-tagged PLD1 isoforms (although the expression of these constructs was very low; results not shown). The localization of these isoforms was independent of the level of protein expression in transfected cells and did not appear to alter due to fixation and treatment of the cells for microscopic analysis (results not shown). These experiments were also repeated in HEK-293 cells, where it was also evident that the GFP–PLD1a and GFP–PLD1b constructs produced fluorescence on vesicular structures (despite the heterogeneous morphology of HEK-293 cells).

To determine the nature of these PLD1a- and PLD1b-containing vesicular structures HeLa cells were transiently transfected with GFP–PLD1a or GFP–PLD1b to monitor colocalization with a variety of organelle markers. It was observed that EEA1, a Rab5 GTPase effector protein found in early endosomes [25], appears on vesicles of a similar size and distribution to those seen for GFP–PLD1a and GFP–PLD1b, and that significant overlap and colocalization occurred between GFP–PLD1a and GFP–PLD1b and EEA1 (Figure 1). There was no detectable colocalization between GFP–PLD1a or GFP–PLD1b and a number of other cellular markers, including the

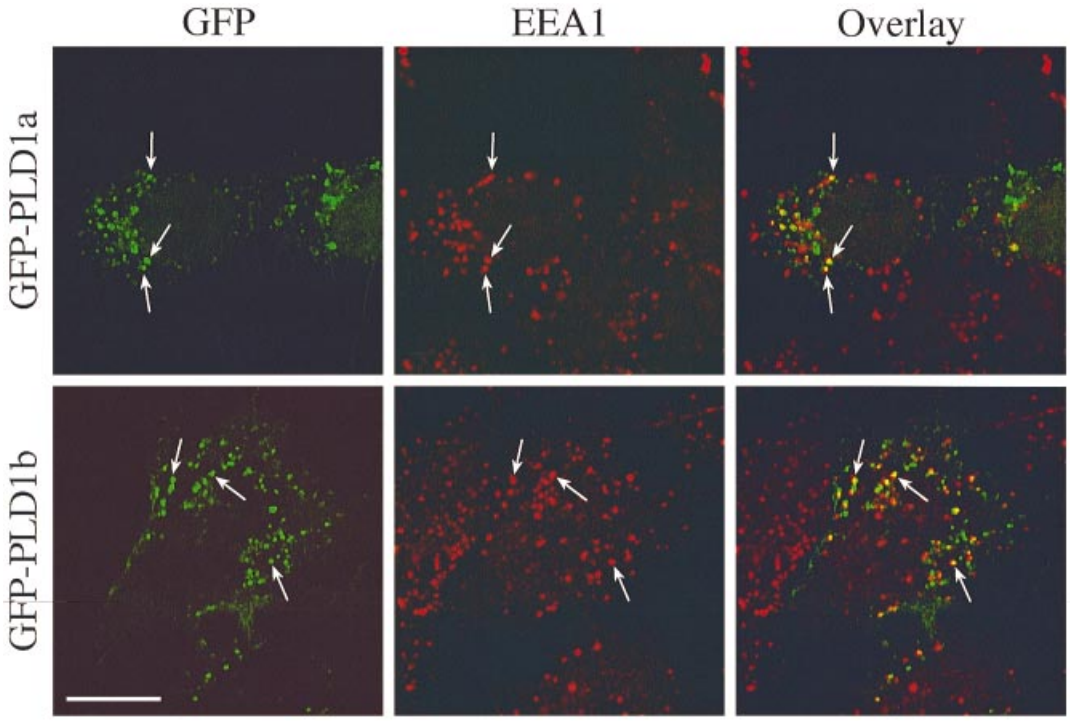

Figure 1 Cellular localization of PLD1a and PLD1b, and colocalization with EEA1

HeLa cells grown on coverslips were transiently transfected with GFP–PLD1a or GFP–PLD1b for 24 h before preparation for microscopy. Representative confocal images of cells expressing GFP–PLD1a or GFP–PLD1b (green) also stained with antibodies raised against EEA1 (red) are shown. Each channel is shown separately and a merged image shows colour change (to yellow) for colocalization. Examples of specific vesicles are indicated with arrows. The bar represents 25 μm.

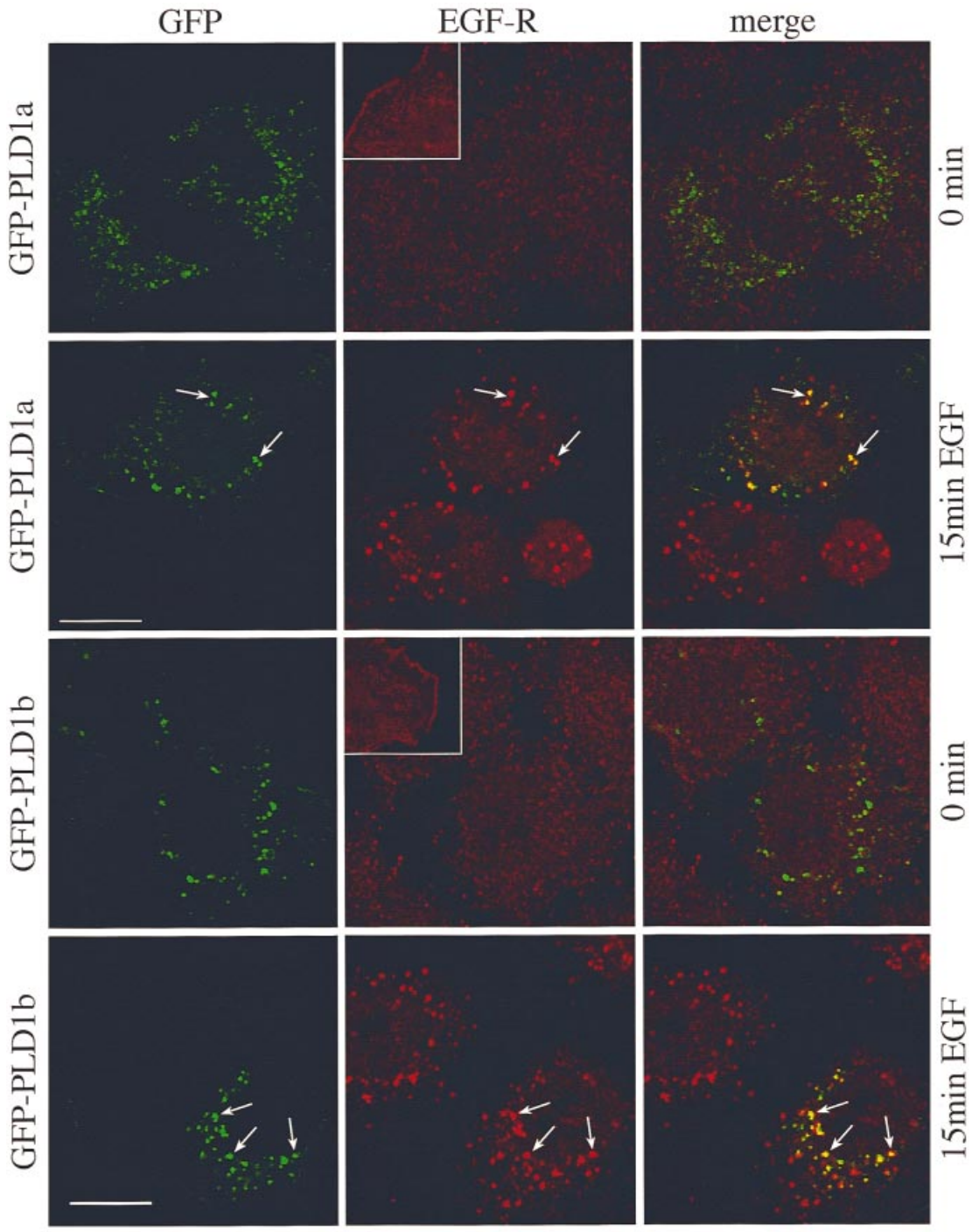

Figure 2 Cellular localization of PLD1a and PLD1b, and colocalization with internalizing EGF-R

HeLa cells grown on coverslips were transiently transfected with GFP–PLD1a or GFP–PLD1b for 24 h before preparation for microscopy. Representative confocal images of cells expressing GFP–PLD1a or GFP–PLD1b (green) also stained with antibodies raised against EGF-R (red), which were either untreated or stimulated with EGF (100 ng/ml) for 15 min, are shown. Each channel is shown separately and a merged image shows colour change (to yellow) for colocalization. Examples of specific vesicles are indicated with arrows. Note, EGF-R distribution before stimulation is difficult to detect; however, after stimulation EGF-R is concentrated on internalizing endosomes and is readily detected (see the text). The insets show plasma-membrane localization of EGF-R before stimulation, detected by incubating cells with antibody at 4 °C before fixation (see the Experimental section). The bar represents 25 μm.

Golgi proteins p115 [26] and GM130 [27], caveolin-1 [28], clathrin heavy chain [29] and the AP complexes AP-1 and AP-2 [30], which are associated with caveolae and clathrin-coated small vesicles (results not shown).

To verify these observations, colocalization with other endosomal markers was investigated. The EGF-R (ErbB1), is progressively internalized after stimulation with EGF and is predominantly directed from the plasma membrane through

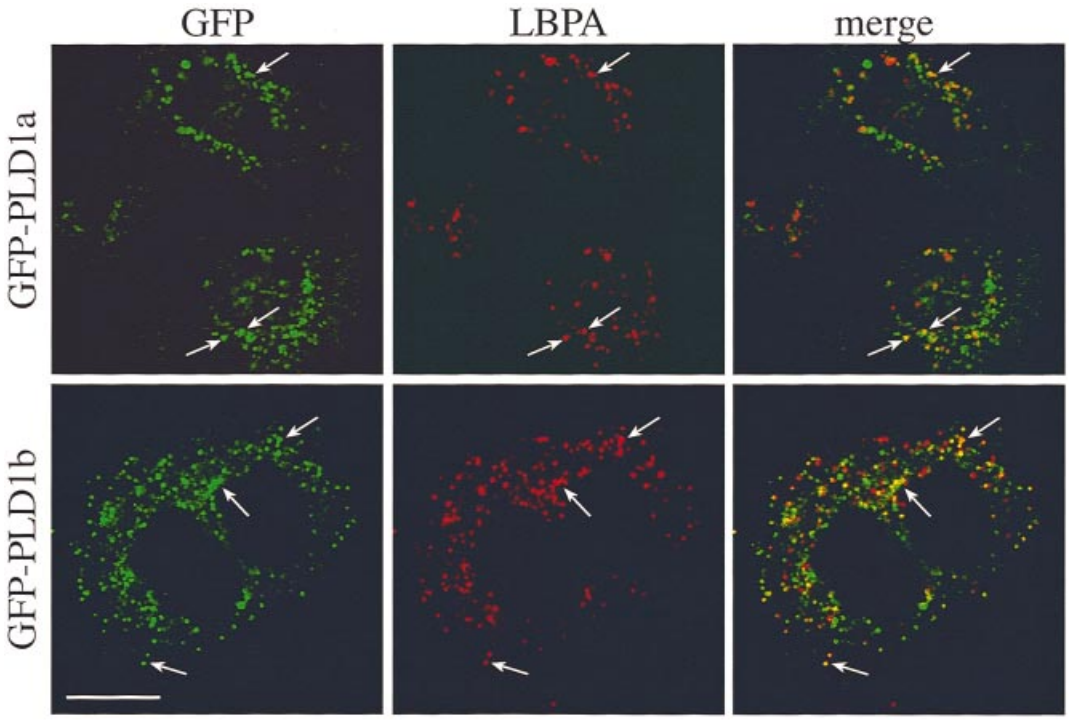

Figure 3 Cellular localization of PLD1a and PLD1b, and colocalization with LBPA

HeLa cells grown on coverslips were transiently transfected with GFP–PLD1a or GFP–PLD1b for 24 h before preparation for microscopy. Representative confocal images of cells expressing GFP–PLD1a or GFP–PLD1b (green) also stained with antibodies raised against LBPA (red) are shown. Each channel is shown separately and a merged image shows colour change (to yellow) for colocalization. Examples of specific vesicles are indicated with arrows. The bar represents 25 μm.

clathrin-coated vesicles, early endosomes and multivesicular bodies to late endosomes for degradation in lysosomes [31,32]. Before stimulation with EGF, cellular EGF-R displays little localization on vesicles in untransfected cells or cells transfected with GFP–PLD1a or GFP–PLD1b, and there was no detectable colocalization between these proteins (Figure 2, 0 min). Indeed, before stimulation, EGF-R localization (predominantly plasma membrane) is actually difficult to detect in HeLa cells. However, upon EGF stimulation EGF-R localization changes dramatically and appears to concentrate, revealing prominent vesicles many of which also contained GFP–PLD1a and GFP–PLD1b (Figure 2, 15 min). Many of these GFP–PLD/EGF-R vesicles also colocalize with EEA1 confirming that these structures are early endosomes (results not shown). Over a time course of EGF stimulation maximum colocalization between EGF-R and GFP–PLD1 or GFP–PLD1b appeared to occur after 15 min of stimulation (results not shown). To confirm this observation the colour of pixels in retrieved confocal images was assessed to quantify colocalization of PLD1a and PLD1b and internalized EGF-R. By assessing the percentage of green vesicular pixels, representing GFP–PLD1a or GFP–PLD1b that were also red, representing EGF-R, it appeared that peak colocalization [65.0 % $\pm$ 5.3 ($n = 12$) for GFP–PLD1a and 52.9 % $\pm$ 4.6 ($n = 12$) for GFP–PLD1b] did indeed occur after 15 min of EGF stimulation, consistent with the microscopic observations. A similar analysis for the Golgi protein p115, where no colocalization was observed, gave a background overlap figure of 6 % $\pm$ 11 ($n = 8$) (although this analysis is not directly comparable as Golgi is not vesicular). This is consistent with there being little Golgi PLD1a and PLD1b. A similar quantification analysis of the confocal images for GFP–PLD1a or GFP–PLD1b and EEA1 (see Figure 1) gave values of 20.9 % $\pm$ 2.3 ($n = 8$) and 20.0 % $\pm$ 2.5 ($n = 8$) respectively.

Peak colocalization of internalized EGF-R with EEA1 appeared to occur earlier than 15 min post-stimulation (results not shown), consistent with observations made by others (see [33]). Additionally, since only a proportion of cellular GFP–PLD1a or GFP–PLD1b appears in the EEA1 compartment, colocalization between GFP–PLD1a or GFP–PLD1b and other endosomal markers was sought. In cells expressing representative levels of GFP–PLD no colocalization was seen with the transferrin receptor, which is recycled to the plasma membrane via the recycling endosomes [31], LAMP-1, found in the *trans*-Golgi network (TGN) and lysosomes [34], or the cation-independent mannose-6-phosphate receptor found in the TGN, as well as late endosomes [35]. However, it was found that LBPA, a lipid found on endosomal structures predominantly on the internal membranes of multivesicular bodies [20], also appears on vesicles of a similar size and distribution to those seen for GFP–PLD1a and GFP–PLD1b, and that significant overlap and colocalization could be detected between GFP–PLD1 isoforms and LBPA [Figure 3; analysis of the confocal images for GFP–PLD1a or GFP–PLD1b and LBPA gave figures of 25.0 % $\pm$ 2.6 ($n = 7$) and 47.9 % $\pm$ 4.8 ($n = 9$) respectively]. The implications of this difference in quantification and the differences seen between PLD isoforms and EGF-R, which may represent a difference in the distribution of PLD1a and PLD1b throughout the endosomal pathway, are currently under investigation.

It is surmised that GFP–PLD1a and GFP–PLD1b are not located in the Golgi apparatus, TGN, lysosomes, recycling

endosomes, clathrin-coated vesicles or caveolae in HeLa cells, but are located in endosomal structures, including early and LBPA-containing endosomes.

Non-endosomal splice variant of PLD1

Cloning of PLD sequences in our laboratory has led to the identification of a PLD1 variant with a distinct C-terminus. Reverse-transcriptase PCR confirmed that this was derived from a message(s) comprising all the PLD1 conserved sequences, as summarized in Figure 4(A). To confirm that this PLD1 variant was expressed *in vivo* a peptide specific to the unique protein sequence at the extreme C-terminus was synthesized and polyclonal antisera were raised against the peptide. Additionally, to compare expression of this novel PLD1 variant with PLD1 itself, antisera were also raised against a peptide derived from the C-terminus of PLD1. The resulting antisera were purified and their specificity confirmed against bacterially-expressed N-terminally truncated PLD1 (results not shown). The antisera were then used to test for the presence of PLD1 antigens in extracts from a variety of cell lines, including DX3 and U937 cells in which the variant cDNA was first identified. Both antisera specifically detected antigens of the approximate predicted sizes for PLD1 and the variant in a number of cell lines including B cells (Jurkat), T cells (Bristol8) and U937 monoblastoid cells (results not shown) and, in addition, cervical carcinoma cell lines (HeLa S3 cells, results not shown, and HeLa Ohio cells, see Figure 4B). Although the novel variant PLD1 was PCR-amplified only in the PLD1a form and detected by Western-blot analysis as a single band this does not preclude the existence of the related PLD1b isoform.

To characterize the cellular localization of these splice variant PLD1 enzymes (designated by the suffix 2) it was confirmed that the PLD1 isoforms were expressed in HeLa cells by Western-blot analysis with the splice-specific antisera (Figure 4B), and cells were then transiently transfected with GFP–PLD1a2 and GFP–PLD1b2. Protein analysis by Western blotting using GFP-specific monoclonal antibodies confirmed that full-length GFP–PLD1 isoforms were being produced in these transfected cells (results not shown), and examination by confocal microscopy revealed that GFP–PLD1a2 and GFP–PLD1b2 appear to be distributed quite differently to the localization seen for GFP–PLD1a and GFP–PLD1b (see above). The distribution of GFP–PLD1a2 and GFP–PLD1b2 appears throughout the cell with some regions of exclusion, including the nucleus and other smaller regions (Figure 4C), and they show no colocalization with endosomal markers (results not shown).

All four PLD1 isoforms are located in a membrane fraction

Cytosolic PLD1-like activity has been described in HL60 cells [36] and a variety of cellular tissues [37], and it has been suggested that the cytosolic PLD activity is likely to result from products of the PLD1 gene [11], as both cytosolic and membrane-bound PLD1 activities are activated by ARF [36,38]. To identify whether the PLD1a2 and PLD1b2 isoforms could represent this cytosolic activity, membrane and cytosol preparations from HEK-293 cells transfected with the GFP–PLD1 isoforms were examined. All four PLD1 isoforms appeared exclusively in the membrane fraction, and by Western-blot analysis no GFP–PLD could be detected in the cytosol preparation (Figure 5). Thus PLD1a2 and PLD1b2, like PLD1a and PLD1b, also appear to be membrane-bound PLD isoforms.

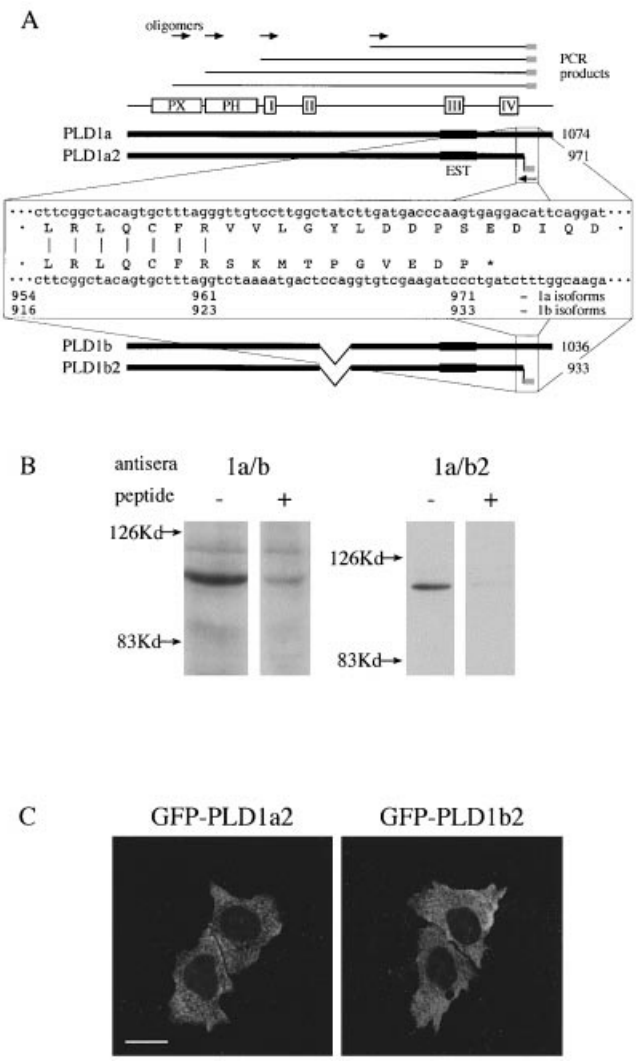

Figure 4 Novel PLD1 isoform

(**A**) Representations of PLD1a and PLD1b and novel splice variants PLD1a2 and PLD1b2 showing approximate positions of the various domains found within mammalian PLD1 isoforms. The PX domain, thought to be involved in protein–protein interactions, the PH domain involved in binding phosphatidylinositol 4,5-biphosphate [44], and the various conserved motifs (I–IV), some of which are involved in the catalytic activity of the protein, are shown (reviewed in [2]). Some of the sequence of PLD1 and the novel splice variation (designated by the suffix 2) is shown within the central box. The stop codon (*) encoded by the novel splice variation (EMBL accession number AJ276230) and the amino acids (numbered) encoded by the DNA for the PLD1 isoforms are also indicated. Lines representing the PCR products obtained from cDNA amplified using splice- and PLD1-specific oligomers (indicated by arrows) are shown at the top of the Figure. (**B**) Extracts from HeLa Ohio cells were resolved by SDS/PAGE (10% polyacrylamide). Following transfer and blocking, antigen was detected using the C-terminal specific PLD rabbit antisera, 1a/b or 1a/b2, by chemiluminescence. Specificity of this detection was confirmed by use of the cognate peptide antigen, which specifically competes for antibody (+, with peptide; −, without peptide). The molecular masses indicated represent the centre position of 126 kDa (Kd) and 83 kDa molecular-mass standard markers. These representative images were obtained from different gels. (**C**) HeLa cells grown on coverslips were transiently transfected with GFP–PLD1a2 or GFP–PLD1b2 for 24 h before preparation for microscopy. Representative confocal images of cells expressing GFP–PLD1a2 or GFP–PLD1b2 (white) are shown. The bar represents 25 μm.

PLD1a2 and PLD1b2 retain phospholipase activity, albeit reduced

To characterize the biochemical properties of these isoforms, PLD activity was assessed in HEK-293 cells grown in DMEM

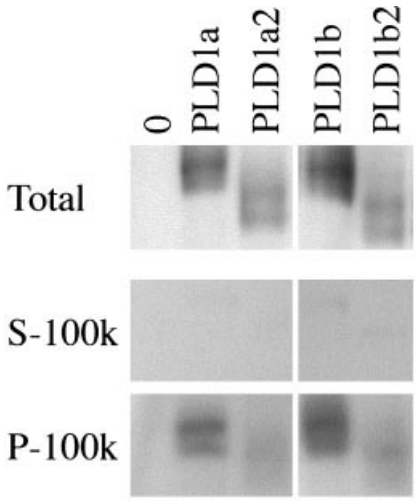

Figure 5 Membrane localization of PLD1 isoforms

HEK-293 cells grown in DMEM supplemented with 10% (v/v) foetal bovine serum were transiently transfected with GFP–PLD1a, GFP–PLD1b, GFP–PLD1a2 or GFP–PLD1b2. After 48 h cells were washed and disrupted by homogenization. A proportion of the total extract (Total) was then separated by centrifugation at 350 000 ***g*** into membrane pellet (P-100k) and supernatant (S-100k) fractions (see the Experimental section). Equivalent samples of each preparation were resuspended in urea sample buffer for transfer to membranes, following resolution by SDS/PAGE (10% polyacrylamide). After blocking, antigen was detected using a GFP-specific antibody by chemiluminescence.

supplemented with 10% (v/v) foetal bovine serum and transiently transfected with one of the four GFP–PLD1 isoforms. Assayed *in vivo* by addition of 2% (v/v) ethanol to cells prelabelled with [^{3}H]myristic acid (which is incorporated into cellular phosphatidylcholine) HEK-293 cells (and cells transfected with empty GFP-vector) produced very little phosphatidylethanol, indicating that the cells have very low endogenous PLD activity. Cells transfected with GFP–PLD1a and GFP–PLD1b showed approximately 20-fold increases in PLD activity; however, cells transfected with the splice variant GFP–PLD1a2 and GFP–PLD1b2 isoforms showed very little increase in phosphatidylethanol production, indicative of modest changes in PLD activity (results not shown). To account for differences in expression of the different GFP–PLD1 isoforms extracts were prepared from similarly transfected cells, and PLD activity was assessed by measuring phosphatidylethanol production from labelled phosphatidylcholine added to the extracts as liposomes. The results of this *in vitro* assay were then related to protein expression determined by Western-blot analysis using GFP-specific antibodies. The expression of all isoforms was approximately equal, and the results shown in Figure 6(A) confirm the initial observation that PLD activity from the C-terminal splice variant isoforms was low, being approximately 8–12% of that seen for GFP–PLD1a and GFP–PLD1b.

To further address the activity of the splice variants, mutants were constructed of the C-terminal splice variant isoforms that contained base substitutions within the proposed catalytic His-Lys-Asp ('HKD') motifs (PLD1b2-K466E and PLD1b2-K860E see [16]). HEK-293 cells were then transfected with wild-type and mutant forms of all PLD1 isoforms to compare *in vitro* PLD activity. All constructs were expressed to an approximately similar level, as assessed by Western-blot analysis, and the results, shown in Figure 6(B), indicate that mutation within the catalytic sites of PLD1b and the C-terminal splice variant PLD1b2 leads to the production of protein with undetectable PLD activity, exhibiting levels equivalent or less than those seen for untransfected cells. Cells transfected with GFP–PLD1b2 consistently showed PLD activity greater than untransfected cells. Additionally, removal of the GTP[S], added to the cell extracts to activate endogenous small GTPases (such as ARF), significantly reduced the activity of all the PLD1 isoforms, including PLD1a2 and PLD1b2 (results not shown), confirming that the isoforms are indeed low activity variants of PLD1. These results concurred with results obtained with PLD1 isoforms expressed in the yeast *Saccharomyces cerevisiae*, where it was

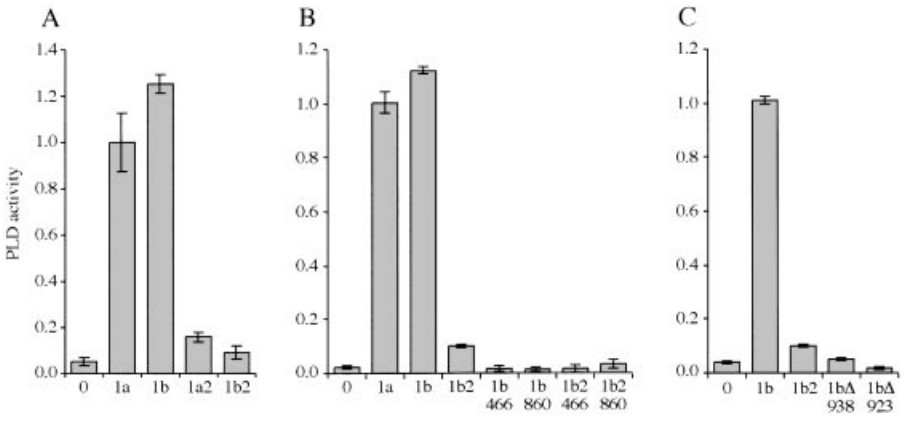

Figure 6 PLD activity of wild-type and mutant PLD isoforms

HEK-293 cells grown in DMEM supplemented with 10% (v/v) foetal bovine serum were untransfected (0) or transiently transfected with (**A**) GFP–PLD1a (1a), GFP–PLD1b (1b), GFP–PLD1a2 (1a2) or GFP–PLD1b2 (1b2), (**B**) GFP–PLD1a, GFP–PLD1b, GFP–PLD1b2 or the point mutants GFP–PLD1b-K466E, GFP–PLD1b-K860E, GFP–PLD1b2-K466E or GFP–PLD1b2-K860E, as indicated, or (**C**) GFP–PLD1b, GFP–PLD1b2, GFP–PLD1b-Δ938 or GFP–PLD1b-Δ923, as indicated. After 48 h extracts were prepared and PLD activity was assessed by determining the ability of extracts to produce labelled [^{3}H]phosphatidylethanol from exogenously added vesicles containing [^{3}H]phosphatidylcholine, a reaction specific to PLD. The activity shown is relative to PLD1a (**A** and **B**) or PLD1b (**C**) and is normalized to GFP–PLD protein detected and quantified after Western-blot analysis (with antibodies to GFP) using densitometry software (see the Experimental section). The levels of GFP–PLD expression measured did not vary by more than 20% of that seen for GFP–PLD1a or GFP–PLD1b. The activity from an equivalent amount of untransfected HEK-293 cells is shown as a percentage of the activity recorded for PLD1a or PLD1b. The error bar represents the range seen between duplicate assays and is representative of two separate experiments.

observed that PLD1a2 and PLD1b2 exhibited ARF-, RhoA- and protein kinase C (PKC)α-activated PLD activity, although activity was approximately 50% of that seen for PLD1a or PLD1b (results not shown). It is unclear why the relative activities of these variants expressed in yeast and mammalian cells are so different; however, it is clear that the mammalian-expressed PLD1a2 and PLD1b2 isoforms retain PLD activity, albeit significantly less than the other PLD1 isoforms. Whether particular agonists may target this specific class of PLD1 to permit expression of full catalytic potential remains to be determined.

C-terminus of PLD1 isoforms is involved in catalysis

The C-terminus of PLD1a and PLD1b has been implicated as an important component involved in the catalytic activity of PLD1. Deletion of the 98 C-terminal amino acids of human PLD1a (PLD1a-Δ976) abolished *in vitro* PLD activity, although this deletion mutant retains some PLD activity (2% of that seen for the wild-type protein) when in the presence of PKCα [16]. Additionally, insertion of two amino acids at position 1028 of PLD1a reduces phospholipase activity to approximately 34% of that seen for the wild-type protein; however, this activity could be increased in the presence of ARF or PKC [16]. Protein containing only the N-terminal half of rat PLD1 exhibited reduced PLD activity and this activity could be restored to wild-type levels by addition of protein consisting of the remaining C-terminal half of the protein, suggesting that the C- and N-termini of PLD1 interact to form an active protein [39]. It has also been demonstrated that deletion of the last four amino acids of rat PLD1 abolishes PLD activity of this protein, although substitution of these amino acids to other charged or less hydrophobic amino acids restored some PLD activity to these proteins and addition of protein with an intact C-terminus restored full PLD activity [40]. The importance of these C-terminal amino acids has been confirmed by a complementary analysis of human PLD1, which also established that PLD activity could be partially restored to deletion mutants by the addition of a peptide consisting of the six C-terminal amino acids [41]. These data would indicate that the C-terminus, absent in PLD1a2 and PLD1b2, should have a role in catalytic activity and therefore that PLD1a2 and PLD1b2 might be predicted to have no PLD activity at all. To investigate this we constructed PLD1 deletion mutants. These consisted of GFP–PLD1a and GFP–PLD1b deleted either at the C-terminal splice site, PLD1a-Δ961 and PLD1b-Δ923, or deleted 15 amino acids distal from the C-terminal splice site, PLD1a-Δ976 and PLD1b-Δ938. These deletion mutants exhibited no detectable PLD activity (Figure 6C; results for PLD1a-Δ961 and PLD1a-Δ976 are not shown), indicating that the C-terminus is indeed important for catalysis and that the short novel C-terminus present in PLD1a2 and PLD1b2 must be sufficient to restore the partial PLD activity detected.

C-termini of PLD1a and PLD1b define precise cellular localization

It has been proposed that the catalytic activity of PLD1 may influence the localization of the protein; indeed, catalytically inactive PLD1a-S911A has been demonstrated to show a cytosolic localization in COS cells [42], although this may reflect changes in the modification of the mutant protein [43]. To assess whether the reduced catalytic activity of GFP–PLD1a2 and GFP–PLD1b2 determined the localization of PLD1 isoforms, HeLa cells were transiently transfected with the catalytic site mutants of PLD1a and PLD1b isoforms. Confocal analysis revealed that cells expressing representative levels of catalytically inactive GFP–PLD1b-K466E or GFP–PLD1b-K860E continue to exhibit a vesicular distribution (Figure 7A). These vesicles remain colocalized with EEA1 and LBPA confirming that these mutants continue to localize to endosomal structures (results not shown). We also examined the distribution of the catalytic mutants in HEK-293 cells and again observed vesicular distribution reminiscent of that seen for wild-type PLD1a or PLD1b. Therefore it appears that inactivation of PLD by mutation within the catalytic HKD motifs does not significantly affect the endosomal localization of PLD1a or PLD1b. As the C-terminal splice variants are not located in endosomes this suggested that the C-terminus may define the endosomal localization of PLD1a and PLD1b in a manner independent of PLD activity. To confirm this, the cellular localization of the PLD1 isoforms deleted from the C-terminus was determined. GFP–PLD1a-Δ961, GFP–PLD1b-Δ923, GFP–PLD1a-Δ976 or GFP–PLD1b-Δ938 continue to exhibit membrane localization, since antigen representing the GFP–PLD deletion mutants could only be detected in a membrane pellet from transiently transfected HEK-293 cells (results not shown). When transfected into HeLa cells the cellular localization of the deletion mutants was dispersed throughout the cell and indistinguishable from that of the PLD1a2 and PLD1b2 splice variants (Figure 7B); these observations were confirmed in HEK-293 cells. This suggests that the C-termini of PLD1a and PLD1b are indeed an important determinant of the endosomal localization of these proteins and as the catalytically inactive mutants continue to show endosomal localization that catalytic activity is not the sole determinant of endosomal localization.

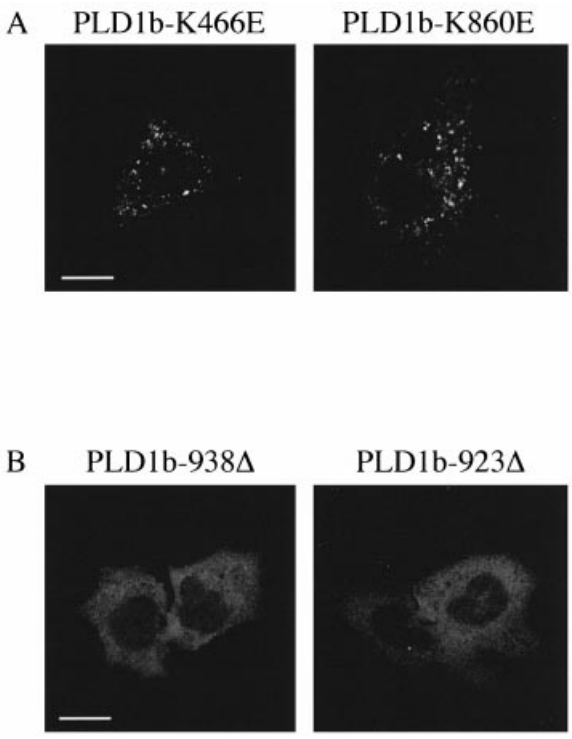

Figure 7 Cellular localization of mutant and deletion PLD1 isoforms

HeLa cells grown on coverslips were transiently transfected with (**A**) GFP–PLD1b-K466E or GFP–PLD1b-K860E, or (**B**) GFP–PLD1b-Δ938 or GFP–PLD1b-Δ923 for 24 h before preparation for microscopy. Representative confocal images of cells expressing GFP–PLD1b-K466E, GFP–PLD1b-K860E, GFP–PLD1b-Δ938 or GFP–PLD1b-Δ923 (white) are shown. The bar represents 25 μm.

Conclusions

It is possible to draw a number of conclusions from the present study regarding the role of the C-termini of PLD1 isoforms. First, the C-termini of the PLD1 isoforms do not influence membrane localization themselves, since all PLD1 mutants and deletions tested were located on cellular membranes. This is consistent with recent observations indicating that other factors may be determinants of membrane localization; the N-terminal PH domain of PLD1 [44] and a central domain of PLD1 [45] have both been shown to bind phosphatidylinositol polyphosphates, and fatty acylation of cysteine residues within the N-terminus of the protein [43] may also influence membrane association. However, the localization of PLD1a and PLD1b in specific membrane compartments does appear to be defined by the C-terminus. Secondly, the C-terminus has a strong influence on the catalytic activity of PLD1. Deletion of the C-terminus destroys PLD1 activity (Figure 6C, [16,39–41,46]); however, it appears that the ten amino acids found in PLD1a2 and PLD1b2 are sufficient to restore partial activity to PLD1a or PLD1b that lack the C-terminal 113 amino acids. However, catalytic activity does not appear to influence endosomal localization. Finally, it is clear that PLD1a and PLD1b are located in endosomes overlapping with compartments containing EEA1, internalized EGF-R and LBPA. Colocalization with the lipid LBPA further defines the localization of these PLD isoforms, but also raises an intriguing possibility. The ability of PLD isoforms to *trans*-phosphatidylate primary alcohols is not restricted to ethanol and butanol (as used in assays for PLD activity) but includes more complex alcohols and polyols such as ethane-1,2-diol (ethylene glycol), butan-1,3-diol and glycerol (propan-1,2,3-triol; W. E. Hughes, unpublished work). Human PLD1 isoforms can also *trans*-phosphatidylate cellular derivatives of glycerol, such as diacylglycerol, to produce bisphosphatidic acid, a potential precursor for LBPA. Production of bisphosphatidic acid by PLD is a property that has been observed *in vitro* (W. E. Hughes, unpublished work) and *in vivo* [47,48]. This potential function for PLD1 isoforms remains to be clarified. Nevertheless, the conclusions from the present study are that in HeLa cells PLD1a and PLD1b are in endosomal compartments and that this is dictated by their C-termini.

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