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Identification of a novel, Ca^{2+}-dependent phospholipase D with preference for phosphatidylserine and phosphatidylethanolamine in *Saccharomyces cerevisiae*

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Abstract A membrane-bound phospholipase D (PLD) from *Saccharomyces cerevisiae* was solubilized from mitochondrial and plasma membranes and partially purified. The enzyme has an apparent molecular weight of approximately 60 kDa, is strictly Ca^{2+}-dependent and preferentially hydrolyses phosphatidylserine and phosphatidylethanolamine. Enzyme activity is significantly increased in membranes from cells grown on a non-fermentable carbon source. The Ca^{2+}-dependent PLD is distinct from PLD encoded by the *SPO14/PLD1* gene. The 195 kDa *SPO14/PLD1* gene product is specific for PtdCho, Ca^{2+}-independent and is activated by PIP_2. Furthermore, Pld1p has transphosphatidylation activity in the presence of ethanol and thus resembles the prototypic PLD activity found in mammalian cells and plants. In contrast, the Ca^{2+}-dependent PLD described here is not affected by PIP_2 and does not catalyze transphosphatidylation. Thus, the Ca^{2+}-dependent PLD characterized in this study appears to be a member of a novel family of phospholipases D.

Key words: Phospholipase D; *Saccharomyces cerevisiae*; Yeast; Phospholipid; Transphosphatidylation; *PLD1*; Calcium; Signalling

1. Introduction

Phospholipases D (PLD, EC 3.1.4.4) are ubiquitous enzymes capable of cleaving phospholipids into phosphatidic acid (PtdOH) and a polar head group alcohol. Both products are potential stimuli of various cellular reactions including DNA synthesis and activation of phosphorylase kinase [1,2]. PtdOH serves as a precursor of diacylglycerol and lyso-PtdOH, which are agonists in signal transduction cascades [3,4]. PLD in mammalian cells can be activated by protein kinase C and via receptor coupled induction responding to numerous agents, such as growth factors and hormones [5].

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Abbreviations: PLD, phospholipase D (EC 3.1.4.4); Ca-PLD, Ca^{2+}-dependent phospholipase D; Pld1p, product of the *PLD1* gene; PKC, protein kinase C; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; PtdIns, phosphatidylinositol; PtdOH, phosphatidic acid; PtdEt, phosphatidylethanol; NBD-PtdCho, 1-palmitoyl-2-[6-*N*-(7-nitro-2-oxo-1,3-benzodiazol-4-yl)-amino]caproyl-*sn*-glycero-3-phosphocholine; PIP_2, phosphatidylinositol-4,5-bisphosphate; PMSF, phenylmethanesulfonylfluoride; EDTA, ethylenediaminetetraacetic acid; PM, plasma membrane

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PLD in the yeast *Saccharomyces cerevisiae* was suggested to be involved in the disintegration of mitochondria in cells undergoing glucose repression [6,7]. Recently a PLD that is induced during sporulation was identified in yeast [8–11]. This yeast PLD is able to perform transphosphatidylation and accepts primary alcohols with chain lengths ranging from one to seven carbon atoms. Transphosphatidylation is a well known reaction for mammalian, plant, and bacterial PLsD [12,13]. The yeast PLD is encoded by a gene previously identified as *SPO14* [14] and in some regions has up to 76% sequence identity with a plant PLD from *Ricinus communis* [15]. Homozygous diploid disruption mutants of *PLD1/SPO14* have no apparent growth phenotype on glucose medium but they are defective in meiosis and are, therefore, unable to complete sporulation [14]. The yeast *PLD1* gene when expressed in a heterologous system, Ci5 cells, gives rise to a membrane associated enzyme, which has substrate specificity for phosphatidylcholine (PtdCho) and is stimulated by phosphatidylinositol-4,5-bisphosphate (PIP_2). Activity is reduced in the presence of Mg^{2+} and oleate, but is unaffected in the presence of the chelators EDTA and EGTA and is not significantly stimulated by Ca^{2+} [9].

Here we report on the identification of a novel, strictly Ca^{2+}-dependent PLD in *S. cerevisiae* that is distinct from Pld1p. The enzyme is membrane bound and shows substrate preference for phosphatidylethanolamine (PtdEtn) and phosphatidylserine (PtdSer). PtdCho is hydrolysed at a 10-fold reduced rate. In contrast to known PLD enzymes this novel yeast PLD is devoid of transphosphatidylation activity.

2. Materials and methods

2.1. Yeast strains and growth media

The following wild type strains of *S. cerevisiae* were used: W303D, *MATa/MATa ura3-1/ura3-1 leu2-3,112/leu2-3,112 ade2-1/ade2-1 his3-11,15/his3-11,15 trp1-1/trp1-1 can1-100/can1-100* (R. Rothstein) and YPH274, *MATa/MATa ura3-52/ura3-52 lys2-80*^{amber}*/lys2-80*^{amber} *ade2-101*^{ochre}*/ade2-101*^{ochre} *trp1-Δ1/trp1-Δ1 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1* [16]. Cells were cultivated at 30°C either on YPD (2% glucose, 2% peptone (Difco), 1% yeast extract (Difco)) or on semisynthetic growth medium [17], containing 2% lactate as the carbon source.

2.2. Disruption of PLD1

PLD1 disruption was carried out by a two-step PCR approach [18] in the diploid strains W303D and YPH274. Using appropriate flanking primers, 98% of the reading frame (bases +15 to +4944) were eliminated and replaced by the *LEU2* selectable marker. Haploid disruption mutants *pld1::LEU2* were obtained after sporulation (0.5% potassium acetate, 0.02% raffinose media), and tetrad dissection.

2.3. Preparation of subcellular fractions

Cell homogenization was carried out in lysis buffer (20 mM Tris-HCl, 0.5 mM EDTA pH 7.5, 1 mM PMSF) with glass beads in a

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Merckenschlager homogenizer (Braun Melsungen). The homogenate was cleared from cell debris and glass beads by centrifugation at 700×*g* for 5 min. For preparation of total membranes cell homogenate was centrifuged at 150000×*g* for 1 h. The supernatant was removed (soluble fractions) and the pellet was homogenized in lysis buffer. Plasma membrane (PM) was prepared by the method of Serrano [19], mitochondria were isolated from spheroplasted cells [17]. The latter protocol was also used for the preparation of microsomes by successive steps of centrifugation at 30000×*g* for 30 min and 150000×*g* for 1 h. The 150000×*g* supernatant was collected ('cytosolic fraction'). Cross-contamination of PM and mitochondria was determined by Western blotting using antibodies against porin (outer mitochondrial membrane marker) and plasma membrane ATPase.

2.4. Assay of phospholipase D activity

PLD activity was determined in an assay system containing 20 mM HEPES pH 7.2, 150 mM NaCl in the presence or absence of 2 mM EDTA, 1 mM $CaCl_2$, 350 mM ethanol or PIP_2, and phospholipid substrate. Protein concentration was typically between 0.1 and 0.5 mg/ml. Radioactively labelled phospholipid substrates were prepared from yeast incubated with [1-^{14}C]palmitate for 2 h [20]. The phospholipid classes were separated by thin-layer chromatography [21]. Purity was >95%. Lyso-phospholipids were prepared from labeled yeast phospholipids by hydrolysis with PLA_2 from *Naja naja* (Sigma Chemicals Inc.). 1,2-Dipalmitoyl-*sn*-glycero-3-phospho[*N*-methyl-^{3}H]choline was obtained from New England Nuclear. Phospholipid-Triton X-100 mixed micelles were prepared by combining phospholipid and detergent dissolved in chloroform/methanol (2/1, v/v), followed by evaporation of the solvent under a stream of nitrogen and subsequent dispersion in water [22]. Substrate and detergent concentration in the assay were 50 μM and 200 μM, respectively. The fluorescent PtdCho analog 1-palmitoyl-2-[6-*N*-(7-nitro-2-oxo-1,3-benzodiazol-4-yl)amino]caproyl-*sn*-glycero-3-phosphocholine (NBD-PtdCho, Avanti Polar Lipids) was dried in vacuum and suspended in H_2O to give a final concentration of 400 μM [11]. PIP_2 (4 mol%) was added as indicated. Enzymatic reactions were started by the addition of the substrate to the assay mixture and stopped after incubation for 30 min at 30°C by the addition of 4 volumes of chloroform-methanol (2/1, v/v) and HCl to a final concentration of 0.1 M. Aliquots of the organic phase were applied to TLC plates and separated with methyl acetate/*n*-propanol/chloroform/methanol/0.25% aqueous potassium chloride (25/25/28/10/7, by volume) [21]. Radioactivity in lipid spots was quantitated using an automatic TLC-linear analyzer Tracemaster 20 (Berthold). Fluorescence was measured with the dual-wavelength Chromato Scanner CS-930 (Shimadzu) with fluorimetric attachment. The reactions were linear for at least 60 min.

2.5. Partial purification of phospholipase D

Gel filtration chromatography: PLD was solubilized from PM or mitochondrial membrane preparations (5 mg protein/ml) with 1 M KCl, 10 mM Tris-HCl pH 7.5, 0.1 mM PMSF on ice for 1 h. After centrifugation at 150000×*g* for 45 min 2 ml aliquots were applied to a Sephacryl S-200 column (1.6×100 cm, Pharmacia) equilibrated with 1 M NaCl, 10 mM Tris-HCl pH 7.5. Proteins were eluted with the same buffer at a flow rate of 25 ml/h at 4°C. Fractions (3 ml) were collected and assayed for PLD activity.

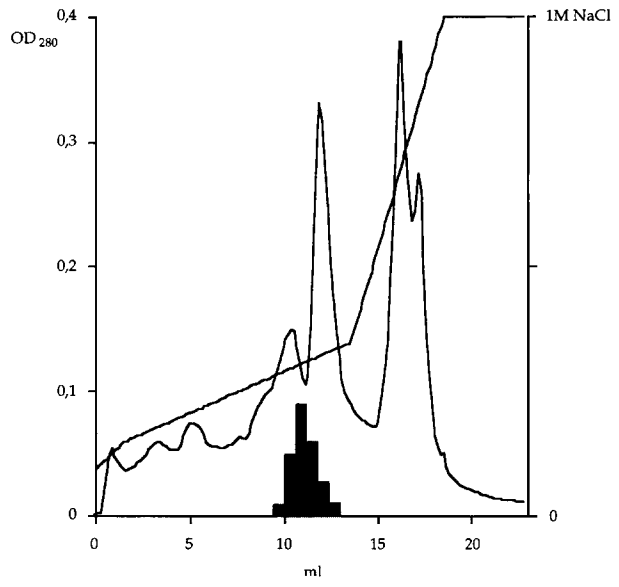

Fig. 1. Elution of Ca-PLD from a mono Q ion exchange column with a NaCl gradient. Black bars indicate Ca-PLD activity.

Ion exchange chromatography: PLD was solubilized from mitochondrial membrane preparations (5 mg/ml) with 0.5% sodium cholate, 10 mM Tris-HCl pH 8.0, 0.1 mM PMSF on ice for 1 h. After centrifugation at 150000×*g* for 45 min, 50 ml aliquots were applied to a column (1.6×15 cm) of Q-Sepharose Fast Flow (Pharmacia) equilibrated with 0.5% sodium cholate, 10 mM Tris-HCl pH 8.0. Proteins were eluted with 150 ml equilibration buffer at a flow rate of 4 ml/min followed by a linear gradient with 0–1 M NaCl in equilibration buffer. Fractions (5 ml) were collected and assayed for PLD activity. The two fractions containing highest PLD activity were diluted 1:5 with equilibration buffer and applied to a Mono-Q-column (Pharmacia). Elution was carried out with a linear gradient of NaCl (0–1 M) in equilibration buffer. 0.5 ml fractions were collected and assayed for PLD activity.

3. Results

3.1. Identification and subcellular localization of Ca^{2+}-dependent phospholipase D

In the presence of 1 mM $CaCl_2$ at pH 7.5 the formation of choline and PtdOH from PtdCho was observed with yeast

Table 1
Partial purification of the Ca^{2+}-dependent phospholipase D from *S. cerevisiae*

Purification step	Protein (mg)	Specific activity (nmol min^{-1} mg^{-1})	Purification factor	Yield (%)
Method I (detergent solubilization)				
Homogenate		0.06		
Mitochochondrial membranes	250	0.15	1	100
0.5% Na-cholate solubilization	112	0.22	1.5	65
Q-fast flow	7.5	2.0	14	41
Mono Q	0.9	12.5	84	29
Method II (salt solubilization)				
Plasma membrane	10	0.4	1	100
1 M KCl solubilization	5.1	0.56	1.4	71
Sephacryl S-200	0.4	2.2	5.6	23

Method I uses solubilization with 0.5% Na-cholate, method II starts up with 1 M KCl extracts of the plasma membrane. Enzyme activity was determined using PtdEtn-Triton X-100 mixed micelles as the substrate (see Section 2).

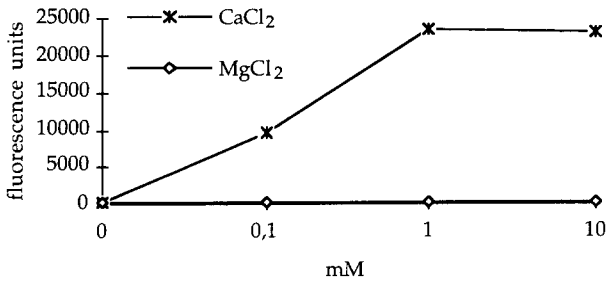

Fig. 2. Effect of Ca^{2+} and Mg^{2+} on the activity of partially purified Ca-PLD, using NBD-PtdCho as the substrate.

homogenate, suggesting cleavage by a Ca^{2+}-dependent PLD activity (referred to as Ca-PLD). Ca-PLD activity was associated with membranes and absent from soluble fractions. In the presence of 1 M KCl Ca-PLD was stimulated three-fold and equivalent amounts of ^{14}C-labeled PtdOH and ^{3}H-labeled choline were released from doubly labeled PtdCho. In the absence of salt higher amounts of choline were found, compared to PtdOH. Since equivalent amounts of diacylglycerols were identified in the assay mixture, this result is most likely due to phosphatidate phosphatase-catalyzed breakdown of PtdOH to diacylglycerol [23]. With PtdEtn as the substrate highest Ca-PLD specific activity (0.4 nmol min^{-1} mg^{-1}) was found in the plasma membrane. Specific activity was significantly lower in mitochondria (0.12 nmol min^{-1} mg^{-1}) and only 0.04 nmol min^{-1} mg^{-1} in the microsomal fraction. Ca-PLD activity, however, was increased 2–3-fold in total membranes prepared from lactate grown cells.

3.2. Partial purification of Ca^{2+}-dependent PLD

Solubilization of Ca-PLD from membranes was achieved either with high salt concentrations (0.5–2 M KCl) or sodium cholate. When salt extracts were subjected to gel filtration chromatography, Ca-PLD activity eluted in a single peak corresponding to a molecular mass of 60 ± 15 kDa (not shown). Sodium cholate extracts of mitochondrial membranes were subjected to two steps of anion exchange chromatography which yielded 84-fold enrichment (Table 1 and Fig. 1). Further attempts to purify the enzyme by additional chromatographic steps resulted in loss of enzyme activity which was probably due either to instability of the pure enzyme or to separation from activating substances.

3.3. Ca^{2+} dependence and substrate specificity

Partially purified PLD has a pH optimum between 7 and 8 [24]. As shown in Fig. 2, the enzyme strictly depends on Ca^{2+} which could not be replaced by Mg^{2+}, Ba^{2+} or Zn^{2+}. Maximum activity was obtained at 1 mM, half maximal activating concentration was 0.1 mM Ca^{2+}. Presence in the assay of 0.2 mM 1,10-phenanthroline had no effect. Partially purified enzyme showed a preference for PtdSer (22 nmol min^{-1} mg^{-1}) and PtdEtn (12 nmol min^{-1} mg^{-1}), PtdCho was hydrolysed at a 10-fold lower rate compared to PtdEtn (Table 2). With PtdCho extracted from yeast or NBD-PtdCho as the substrate and 2% ethanol partially purified Ca-PLD did not catalyze transphosphatidylation. PtdIns, lyso-PtdCho or lyso-PtdEtn were not used as substrates.

3.4. Phospholipase D activity in pld1 mutants

Recently, the *PLD1* gene encoding a PLD was cloned and sequenced [9–11]. Pld1p, as derived from the sequence, has a predicted molecular weight of 195 kDa. This is in marked contrast to the size of Ca-PLD of approximately 60 kDa identified in our studies. Furthermore, Pld1p was independent of Ca^{2+} whereas the PLD described here strictly depended on Ca^{2+}. This discrepancy prompted us to investigate PLD activity in a Pld1p-depleted mutant strain.

Haploid and diploid *pld1* disruption mutants did not show any abnormal growth phenotype on YPD or lactate medium. Homozygous diploids of *pld1* were sporulation-deficient. In *pld1* mutants Ca^{2+}-dependent activity was comparable to that in wild type cells (Fig. 3), demonstrating that yeast contains at least two independent phospholipases D. With yeast PtdCho or POPC as the substrate, PLD activity was low in membrane fractions from the wild type cells incubated in the presence of EDTA and disappeared in membranes from *pld1* mutants. Cleavage of NBD-PtdCho by Pld1p is strongly stimulated by PIP_2 (4 mol%), which is in accordance with previous reports [9,11]. Ca-PLD is not affected by PIP_2 (Fig. 3B).

With membrane preparations from wild type cells, phos-

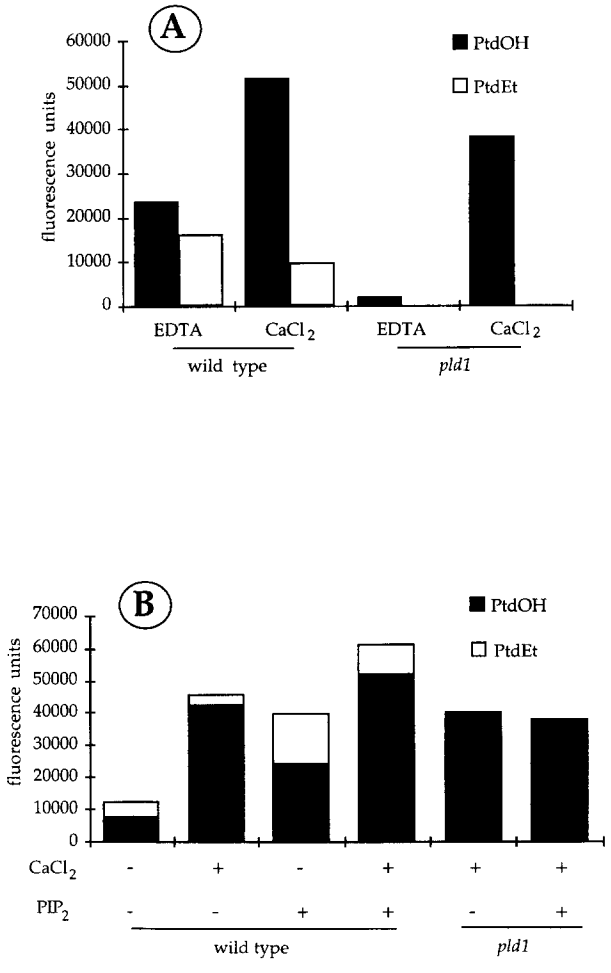

Fig. 3. Phospholipase D activities in the plasma membrane. Enzyme activity was measured using NBD-PtdCho as the substrate (black bars); EDTA was added at a concentration of 2 mM, $CaCl_2$ at 1 mM. A: Activity in preparations from wild type and the *pld1* mutant strain. B: Effect of PIP_2 (4 mol% of NBD-PtdCho) on PLD activity in wild type and *pld1* mutant strains. Transphosphatidylation (white bars) was measured in the presence of 2% (by volume) ethanol.

Table 2
Substrate specificity of the calcium-dependent phospholipase D

Enzyme source	PtdCho	PtdEtn	PtdSer	PtdIns
Na-cholate solubilized protein	0.03	0.22	0.17	n.d.
Fraction from Mono Q column	0.87	12	22	n.d.

Enzyme activity is given in nmol min^{-1} mg $protein^{-1}$. Phospholipids were presented at a concentration of 50 μM in the form of Triton X-100 mixed micelles (n.d., not detectable).

phatidylethanol (PtdEt) was formed in the presence of NBD-PtdCho and 2% ethanol. Highest transphosphatidylation activity in the presence of EDTA was found in the plasma membrane, only at 30% in mitochondria and less than 10% in microsomes. Transphosphatidylation activity was completely lacking in *pld1* mutant extracts. In the presence of Ca^{2+} transphosphatidylation in wild type appeared reduced (Fig. 3B), most likely due to the rapid degradation of PtdEt by the more active Ca-PLD. In fact, NBD-PtdEt was a substrate for Ca-PLD in extracts from *pld1* mutant strains (data not shown).

4. Discussion

In yeast membrane extracts, a novel Ca^{2+}-dependent phospholipase D was identified, and partially purified. This enzyme differs from the only known PLD in yeast and also from enzymes isolated from other sources by its strict Ca^{2+} dependence and lack of transphosphatidylation activity. Thus, the Ca^{2+}-dependent PLD from yeast obviously represents a member of a new family of PLsD. This notion is also supported by the lack of any other sequences homologous to the *PLD1/SPO14* gene in the now completely sequenced yeast genome.

Despite their different properties, Pld1p and Ca-PLD share a similar subcellular distribution which, however, is similar to that of PLD activity in human 3T3 Ci cells. In these cells bradykinin- and phorbol ester-stimulated PLD activity is distributed between the plasma membrane and a fraction consisting of mitochondria and the endoplasmic reticulum [25]. Ca-PLD from yeast can be solubilized with buffers containing high salt concentrations and is thus likely to be a peripheral membrane protein. Below 0.2 M KCl the enzyme tends to precipitate, suggesting also a rather hydrophobic character. Ca-PLD activity is stimulated by solubilizing concentrations of salt or ionic detergents, using 'natural' phospholipids extracted from yeast as the substrate. This effect was not observed with NBD-PtdCho as the substrate. Due to the presence of a short chain and rather hydrophilic fatty acid the latter phospholipid differs from natural phospholipids in its biophysical properties and is thus more easily accessible to the membrane-bound enzyme.

The pronounced selectivity of Ca-PLD for PtdEtn and PtdSer is a striking feature of this enzyme. Since PtdSer (33% of total phospholipids) and PtdEtn (20%) are highly enriched in the plasma membrane [26], this preference for aminophospholipids might be of physiological significance. Activity towards PtdEtn has been reported for PLsD from mammalian and plant sources [27,28], while PtdSer has not yet been described to be a substrate for eukaryotic PLsD. The enzyme does not accept lyso-PtdEtn and lyso-PtdCho in vitro which rather excludes a direct involvement in the generation of lyso-PtdOH from lyso-phospholipids. Colocalization of both phospholipases D in the plasma membrane suggests that these enzymes may be involved in signal transduction pathways signalling external stimuli. However, for Pld1p/Spo14p this appears unlikely [9], since cells lacking Spo14p undergo the first steps of meiosis [14]. Since wild type cells are insensitive to external stimuli after having decided for meiosis, it was suggested that Pld1p is activated rather by an, as yet unidentified, internal event.

Transphosphatidylation is a characteristic feature of known PLsD from yeast, plants and mammalian cells. Yeast Pld1p produces PtdEt and PtdOH at a ratio of approximately 1:5 ([9] and own results). Ca-PLD, however, is devoid of this activity. Lack of transphosphatidylation activity in vitro is also reflected in vivo, since yeast cells grown in the presence of ethanol as the carbon source, or under anaerobic conditions producing ethanol at high concentrations, are devoid of PtdEt. The ability of Ca-PLD to degrade PtdEt might point to a protective action by preventing the accumulation of potentially membrane perturbing PtdEt formed by Ca^{2+}-independent Pld1p.

Strict dependence on Ca^{2+} suggests that regulation of Ca-PLD involves PtdIns specific phospholipase C and protein kinase C. Increased activity during growth on lactate in comparison to glucose implies regulation by metabolites. In contrast to mammalian PLsD [29] Ca-PLD is not activated by GTPγS (30 μM) and cytosolic factors (cytosol added up to 1 mg/ml). Thus, stimulation by ADP ribosylation factors seems to be unlikely. The physiological role of Ca-PLD may be similar to that of known PLsD, but representing a member of a novel family of PLsD it may be implicated in cellular processes for which a function of PLsD has not yet been defined.

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