Interaction of phospholipase D1 with a casein-kinase-2-like serine kinase

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Phospholipase D (PLD)1 was phosphorylated *in vivo* and by an associated kinase *in vitro* following immunoprecipitation. Both phosphorylation events were greatly reduced in a catalytically inactive point mutant in which the serine residue at position 911 was converted into alanine (S911A). The kinase could be enriched from detergent-extracted brain membranes and bind and phosphorylate PLD1 that was immunoprecipitated from COS-7 cells. Using in-gel kinase assays we determined that the size of the kinase is approximately 40 kDa and that PLD1 is more effective than S911A in binding the kinase. Preliminary analysis of the phosphorylation sites on PLD1 suggested that the kinase belongs to the casein kinase 2 (CK2) family. Consistent with this, we found that the kinase could utilize GTP, and could be inhibited by heparin and 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB). Membrane fractions from Chinese hamster ovary (CHO) cell lines that inducibly express PLD1 contained

an endogenous kinase activity that phosphorylated PLD1 using GTP and was inhibited by DRB. Direct evidence that the kinase is CK2 came from observations that immunoprecipitates using PLD1 antibodies contained immunoreactive CK2 α , and immunoprecipitates using CK2 α antibodies contained immunoreactive PLD1. Co-expression of PLD1 in COS-7 cells with the two recombinant CK2 subunits, α or β , suggests that the association of PLD1 with the kinase is through the β subunit. Supporting this, phosphorylation of PLD1 by purified recombinant CK2 α was enhanced by purified recombinant CK2 β . Assays measuring PLD1 catalytic activity following phosphorylation by CK2 suggest that this phosphorylation event does not influence PLD1-mediated hydrolysis of phosphatidylcholine *in vitro*.

Key words: phospholipases, protein interaction, phosphorylation.

INTRODUCTION

The regulation of phospholipase D (PLD) activity in mammalian cells is complex, and involves protein activators that are themselves implicated in the control of diverse cellular pathways [1]. PLD1 is activated by ADP ribosylation factor (ARF), which has defined roles in intracellular trafficking events. Similarly, conventional protein kinase C (PKC) isoforms that regulate pathways influencing cell growth and differentiation also activate PLD1. Finally, activation of PLD1 by the Rho family of small GTP-binding proteins provides a connection between PLD activity and transformation [2], as well as a potential role in the control of cytoskeletal rearrangements. In principle, it is not necessary to assume that the divergence of these activators suggests an important role for PLD in linking membrane homeostasis to multiple facets of cell function. On the other hand, this remains an attractive hypothesis and it would imply the co-ordination of several signal transduction pathways by a relatively small set of molecules [3].

Consistent with a complex set of regulatory requirements, PLD1 also appears to contain several different post-translational modifications. We have reported that fatty acylation of PLD1 is a mechanism for targeting this lipase to intracellular membranes and for modulating its *in vivo* activity [4,5]. In addition, PLD1 can be phosphorylated on tyrosine residues following stimulation by peroxides of vanadate, and such phosphorylation may correlate with enhanced PLD activity [6,7]. Recent reports also

indicate that PLD1 is phosphorylated on serine and threonine residues by PKC while resident on specialized plasma-membrane subdomains termed caveolae [8]. PLD1 also binds to concanavalin A, suggesting that it is glycosylated [9]. The significance of this observation is currently unknown.

In the present study we report that PLD1 is phosphorylated on serine residues under basal conditions, and we describe a novel association of PLD1 with a serine kinase, casein kinase 2 (CK2). The PLD1–CK2 association is evident in crude cellular lysates, as well as with purified components, and it involves both the α and the β subunits of the CK2 heterotetramer. Association and subsequent phosphorylation may play an important role in signalling pathways mediated by PLD1, by stabilizing and correctly localizing the enzyme to its substrate and regulators.

MATERIALS AND METHODS

Cells, plasmids, antibodies and immunoprecipitations

All transfections were carried out in COS-7 cells using expression plasmids as described previously [4]. For PLD1 immunoprecipitations we used N-terminal antibodies raised in our laboratory that were cross-linked to Protein G–Sepharose beads (Pharmacia) using dimethyl pimelimidate. Immunoprecipitations of CK2 were performed using polyclonal antibodies against the α subunit as described previously [10]. Immunoprecipitations were carried out for 1 h at room temperature, followed by four

Abbreviations used: ARF, ADP ribosylation factor; CHO, Chinese hamster ovary; CK2, casein kinase 2; DMEM, Dulbecco's modified Eagle's medium; DRB, 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole; DTT, dithiothreitol; GST, glutathione S-transferase; IPTG, isopropyl β -D-thiogalactoside; MBP, myelin basic protein; PKC, protein kinase C; PLD, phospholipase D.

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washes in IP buffer [50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.1 % Tween-20 and 0.02 % sodium azide] and two washes in PBS.

Assay for kinase activity on beads

The beads from the final immunoprecipitation wash were collected by brief centrifugation and were incubated in buffer {PBS (pH 7.0) containing 10 mM MgCl₂ and 0.5 μ Ci of [γ -³²P]ATP or [γ -³²P]GTP, as indicated} for 30 min at 37 °C with agitation. Variations to this protocol included the addition of 20 μ M ATP and the addition of 0.3 mg/ml myelin basic protein (MBP) or 0.3 mg/ml histone H1 as reporters of kinase activity. Following the 30 min incubation, Laemmli sample buffer [20 mM Tris/HCl (pH 6.8), 2 % (w/v) SDS, 10 % (v/v) glycerol, 0.1 M dithiothreitol (DTT) and 0.1 % Bromophenol Blue] was added, and the samples were analysed by SDS/PAGE.

In-gel kinase assay

We modified published protocols as follows [11]. Histone H1 (0.5 mg/ml) was added to the running-gel solution prior to polymerization. Gels were loaded and run normally. At the end of electrophoresis, SDS was removed by incubating the gels in 50 mM Tris/HCl (pH 8.0) containing 20 % (v/v) propan-2-ol for 60 min at room temperature with one wash. Denaturing solution [50 mM Tris/HCl (pH 8.0), 6 M guanidine/HCl, 20 mM DTT and 2 mM EDTA] was added for 1 h at room temperature. The gel was then transferred to renaturing solution [50 mM Tris/HCl (pH 8.0), 1 mM DTT, 2 mM EDTA and 0.04 % Tween-20] for 16 h at 4 °C with several changes of solution, but no agitation. For the kinase assay, the gel was equilibrated in buffer [40 mM Hepes (pH 8.0), 1 mM DTT, 0.1 mM EGTA and 20 mM MgCl₂] for 30 min at room temperature, and the reaction was subsequently carried out in the same buffer containing 150 μ Ci of [γ -³²P]ATP for 1 h at room temperature. Unbound radioactivity was removed by extensive washing in IP buffer containing 1 % (w/v) sodium pyrophosphate, and the gel was then dried and exposed to film.

Brain membrane preparation

All steps, including centrifugation, were carried out at 4 °C. Deep-frozen sheep brain (180 g) was broken into small pieces and resuspended in ice-cold buffer [30 mM Tris/HCl (pH 7.6), 80 mM NaCl, 2 mM EGTA, 0.5 mM EDTA, 2 mM DTT, 0.2 mM PMSF and 10 μ g/ml each of antipain, pepstatin A, aprotinin and leupeptin] to a volume of 500 ml for 10–15 min until thawed. The tissue was then blended in a Polytron homogenizer, until it reached a fine consistency, followed by centrifugation at 15000 *g* for 30 min. The resulting supernatant was centrifuged for 1 h at 100000 *g*, and the pellet was resuspended in homogenization buffer [20 mM Hepes (pH 7.2), 1 mM EDTA, 0.2 M sucrose and protease inhibitors as above] and homogenized in a steel Dounce homogenizer with 20 strokes. The homogenate was centrifuged at 2000 *g* to remove debris, and the supernatant, which contained membranes, was centrifuged at 100000 *g* for 1 h. The pellet from this spin was resuspended in a total of 20 ml of homogenization buffer, frozen in liquid nitrogen, and stored at –70 °C until ready to use.

Membrane extraction and binding to immunoprecipitated PLD1

Frozen membranes were quickly thawed at 37 °C and diluted 1:3 in lysis buffer [25 mM Hepes (pH 7.5), 5 mM EDTA, 300 mM

NaCl, 1.5 % (w/v) Nonidet P40 and protease inhibitors] for 30 min at 4 °C. The extract was centrifuged at 15000 *g* for 25 min at 4 °C, and the supernatant was incubated with Protein A–Sepharose beads for 30 min at room temperature. Following incubation, the beads and non-specific proteins were removed, and the cleared supernatant was mixed with Protein G beads containing PLD1 that had been immunoprecipitated from COS-7 cells as described above. Typically, 0.5 ml of membrane extract (5 mg/ml) was used per immunoprecipitated PLD1 from one 15-cm dish. Binding was allowed to proceed for 1 h at room temperature, and the beads were subsequently washed four times with IP buffer and twice with PBS.

Labelling of PLD1 *in vivo*

Labelling experiments were performed with transfected COS cells in 6-well plates. For [³⁵S]TranSlabel labelling, the cells were washed three times with Dulbecco's modified Eagle's medium (DMEM) without methionine and cysteine, and were incubated in this medium for 30 min at 37 °C in order to deplete the intracellular pool of methionine and cysteine. Medium containing 200 μ Ci/ml [³⁵S]TranSlabel was added to the cells for 2 h at 37 °C. At the end of the labelling period the cells were processed for immunoprecipitation as described above. Phosphate labelling was performed in a similar manner using phosphate-free DMEM and 1 mCi/ml [³²P]P_i.

Isolation of Golgi-enriched membranes from Chinese hamster ovary (CHO) cells for kinase assays

Continuous CHO cell lines expressing human PLD1 inducibly were obtained in two steps. First, CHO cells were transfected with plasmids encoding the lac repressor, and an appropriate clone with high expression was selected (designated CHO 3.6). Second, CHO 3.6 cells expressing the lac repressor were transfected with plasmids encoding PLD1 under the control of the cytomegalovirus promoter but also including lac repressor binding sites. Details of creating these cell lines and their biochemical characterization (K. Bi, N. T. Ktistakis, C. Wells, S. Yu, M. Manifava, A. Faulkner, P. C. Sternweis and M. G. Roth, unpublished work) can be obtained from N.T.K. on request. Cells were plated such that they were 80 % confluent on the day of harvesting. They were induced with 5 mM isopropyl β -D-thiogalactoside (IPTG) and 7.5 mM sodium butyrate for 18 h at 32 °C prior to harvesting. Homogenization, using a steel Dounce homogenizer, and fractionation, on sucrose step gradients, were carried out as previously described [12]. Golgi-enriched membranes containing the majority of PLD1 protein were collected from the 0.8/1.2 M interface and stored at –70 °C until ready to use. For kinase assays, the membranes were quickly thawed at 37 °C and diluted in buffer [final concentrations: 20 mM Tris/HCl (pH 7.4), 5 mM MgCl₂ and 1 mM DTT] containing 10 μ Ci of [γ -³²P]GTP. The samples were then incubated at 37 °C for 30 min before addition of Laemmli sample buffer and electrophoresis.

Purification of recombinant CK2 α and CK2 β

Transformed BL21 cells expressing the glutathione S-transferase (GST)–CK2 fusion proteins were grown to an attenuation (*D*₆₀₀) of 0.6 in 500 ml of Luria–Bertani medium. The cells were then induced for 3 h with 1 mM IPTG. Extraction of GST–CK2, purification of the fusion protein, and thrombin cleavage to release GST were performed according to an established protocol (Amersham Pharmacia Biotech).

Phosphopeptide sequence analysis

Phosphorylated PLD1a was prepared either from COS-7 cells labelled with [32 P] P_i for 2 h or from immunoprecipitated PLD1a that was incubated *in vitro* with [γ - 32 P]GTP or [γ - 32 P]ATP to become labelled by the endogenous kinase. The signal from *in vivo* labelling was only sufficient to determine that phosphorylation was on serine residues. Analysis of PLD1a, labelled *in vitro*, by HPLC and Edman degradation, as previously described [13], allowed the identification of several potential phosphorylation sites. One site encompassed the two adjacent serine residues (Ser⁶¹⁰ and Ser⁶¹¹ in PLD1a) in the sequence KLFHPSSSESEQLTR (single-letter amino-acid notation has been used). The identity of the peptide was determined by microsequencing and was confirmed in three independent experiments. Since this is a PLD1a-specific site, we determined whether PLD1b was also phosphorylated *in vitro* under our conditions. We found similar levels of phosphorylation of PLD1a and PLD1b (results not shown). Because of this result, we did not assign functional significance to the phosphorylation at Ser⁶¹⁰ and Ser⁶¹¹, but simply used this information to deduce the identity of the kinase.

Exogenous substrate assay of PLD activity

PLD activity in PLD1 immunoprecipitates was measured essentially as described previously [14]. PLD1 immunoprecipitates from COS-7 cells were incubated for 30 min at 37 °C in buffer {PBS (pH 7.0) containing 0.5 μ Ci of [γ - 32 P]GTP} plus 0.2 μ g each of purified bacterially expressed CK2 α and β subunits in the presence or absence of 10 mM MgCl₂. The immunoprecipitates were then washed to remove excess nucleotide, detergent and CK2 protein. The PLD assay reactions were incubated for 30 min at 37 °C with 10 μ M guanosine 5'-[γ -thio]triphosphate.

RESULTS

In vivo phosphorylation of PLD1

Following methionine labelling of COS-7 cells, overexpressed PLD1 was evident after electrophoresis as a band of 110 kDa (Figure 1A, lane 2). Similarly, following phosphate labelling, a band of 110 kDa, which matched that obtained with methionine labelling, was present (Figure 1A, lane 4). Phosphate labelling of PLD1 did not require any stimulation by growth factors or hormones, and occurred regardless of whether serum was used during the labelling (results not shown). In parallel experiments, we expressed PLD1 after large-scale transfection into COS-7 cells and determined whether a protein kinase capable of phosphorylating PLD1 associated with the enzyme following immunoprecipitation. When PLD1 was immunoprecipitated from large-scale transfections of COS-7 cells using antibodies to the N-terminal domain of PLD1, it associated with a protein kinase activity (Figure 1B). This activity could phosphorylate PLD1 itself (Figure 1B, lanes 2 and 4), and it could readily phosphorylate MBP supplied exogenously (Figure 1B, lane 2). In addition to PLD1, two other polypeptides were phosphorylated by the kinase, one at 24 kDa and another at 18 kDa. Presently, the identity of these polypeptides is unknown. The presence of a kinase activity in the immunoprecipitates was absolutely dependent on PLD1. Immunoprecipitates from cells transfected with a control vector (Figure 1B, lanes 1 and 3) or immunoprecipitates from cells transfected with PLD1 using irrelevant antibodies (results not shown) did not contain a kinase activity. As an additional control, we immunoprecipitated PLD1 with antibodies against the C-terminal domain of PLD1 and assayed

Figure 1 Phosphorylation of PLD1 *in vivo* and by an associated kinase *in vitro*

(A) COS-7 cells were transfected with a plasmid encoding PLD1 (PLD) or with a control plasmid encoding hygromycin resistance (—). After 42 h, the cells were labelled with either [35 S]TranLabel ([35]-S) or with [32 P] P_i ([32]-P) for 2 h at 37 °C. Following labelling, the cells were lysed, and the lysates were immunoprecipitated with polyclonal antibodies directed against PLD1, resolved by SDS/PAGE and subjected to autoradiography. (B) COS-7 cells were transfected and immunoprecipitated as above. Following immunoprecipitation and extensive washing, the samples were incubated with [γ - 32 P]ATP in the presence or absence of 0.3 mg/ml MBP, as indicated, for 30 min at 37 °C. At the end of this incubation, samples were resolved by SDS/PAGE and subjected to autoradiography.

for the presence of the kinase activity. Both N- and C-terminal antibodies immunoprecipitated the protein kinase activity, and this activity was in direct proportion to the amount of immunoprecipitated PLD1 detected by immunoblotting (results not shown). It should be noted that, although endogenous PLD activity [5] and mRNA (M. Manifava and N. T. Kistakis, unpublished work) can be readily detected in COS-7 cells, it has not been possible to detect endogenous PLD1 protein in immunoprecipitates using a variety of antibodies, including our own. The most likely explanation for this is that PLD1 is expressed at extremely low levels and that overexpression is required for detection.

Phosphorylation of wild-type PLD1 compared with S911A

We compared the phosphorylation of wild-type PLD1 with that of a catalytically inactive point mutant (S911A), which we have demonstrated to differ significantly from the wild-type protein in terms of localization and fatty acylation [4,5]. Both proteins are expressed to similar levels in COS-7 cells (Figure 2A), as shown by immunoblotting. However, significant differences in phosphorylation were evident, both *in vivo* (Figure 2B) and by the associated kinase *in vitro* (Figure 2C). Under both conditions, phosphorylation of the S911A mutant was greatly reduced. We originally hypothesized that the differences in phosphorylation suggested that Ser⁹¹¹ is an important phosphorylation site, but subsequent phospho amino acid analysis did not support this hypothesis (results not shown). In fact, it appears that the differences in *in vitro* phosphorylation can be fully accounted for by a reduced amount of kinase associating with the point mutant (see below). The presence of unlabelled ATP during the phosphorylation reaction reduced the overall level of labelling on

Figure 2 Catalytically inactive point mutant of PLD1 shows reduced phosphorylation *in vivo* and *in vitro*

COS-7 cells were transfected with plasmids encoding either wild-type PLD1 or the catalytically inactive point mutant S911A. The cells were subjected to three different assays 42 h after transfection. A portion was immunoprecipitated and resolved by SDS/PAGE followed by immunoblotting (A). A second portion was labelled with [32 P] P_i for 2 h at 37 °C, followed by immunoprecipitation, SDS/PAGE and autoradiography (B). A third portion was immunoprecipitated and subjected to a kinase assay using [γ - 32 P]ATP, followed by SDS/PAGE and autoradiography (C). Note that the relative amounts of PLD1 and S911A as revealed in (A) are very similar.

Figure 3 Characteristics of *in vitro* phosphorylation of PLD1 and S911A

COS-7 cells were transfected with plasmids encoding PLD1 or the S911A point mutant, as indicated. After lysis and immunoprecipitation, the samples were subjected to a kinase assay in the absence of additional reporters (lanes 1, 2, 7 and 8), in the presence of 0.3 mg/ml MBP (lanes 3, 4, 9 and 10) or in the presence of 0.3 mg/ml histone H1 (his; lanes 5, 6, 11 and 12). Each condition was performed in duplicate, with one set of samples containing unlabelled ATP at 20 μ M (lanes 7–12). The exposure shown in (B) is 10 times as long as that shown in (A).

PLD1 without altering the differences between PLD1 and S911A (Figure 3A, compare lanes 1–6 with lanes 7–12). In addition, the kinase could phosphorylate MBP or histone H1, but we found that relative phosphorylation of histone H1 rather than of MBP reflected the differences in phosphorylation between PLD1 and S911A better (Figure 3A, compare lane 5 with lane 6, and lane 11 with lane 12). Finally, although the kinase could phosphorylate MBP or histone H1, its preferred substrate under our conditions was PLD1 if one considers that there was more than a 50-fold molar excess of MBP or histone H1 over PLD1 in those reactions.

This was most evident under conditions where unlabelled ATP was not present (Figure 3A, compare lanes 1–6 with lanes 7–12).

Enrichment of kinase activity and in-gel kinase analysis

Although a kinase activity was readily apparent after immunoprecipitation, we could not detect any additional non-PLD1/non-IgG related bands by silver staining that could indicate a possible size for this kinase from endogenous sources. In addition, by immunoblotting with antibodies to various kinases and by

Figure 4 Kinase that associates with PLD1 can be enriched from brain membranes and has a size of 40 kDa

(A) COS-7 cells transfected with plasmids encoding PLD1 or hygromycin resistance (—) were lysed and immunoprecipitated using PLD1 antibodies. At the end of immunoprecipitation, half of the sample was mixed with a detergent extract from brain membranes (membr. extract; lanes 3 and 4), whereas the other half was incubated with the buffer used to produce the extracts (lanes 1 and 2). Following incubation at room temperature for 1 h, the samples were washed extensively and subjected to a kinase assay, as described in the Materials and methods section, prior to SDS/PAGE and autoradiography. (B) Samples were prepared from COS-7 transfections as above. At the end of immunoprecipitation a sample from the PLD transfection was kept on ice (lane 5). The rest were incubated with either membrane extracts (lanes 3 and 4), or with buffer alone (lanes 1 and 2) for 1 h and were then extensively washed. At the end of these treatments, all five samples were resolved on 10% polyacrylamide gels that were polymerized with added histone H1. After electrophoresis, samples in the gel were renatured and the gel was incubated with [γ - 32 P]ATP for 30 min. After extensive washing, the gel was dried and processed for autoradiography.

varying the activators or inhibitors of the *in vitro* kinase assay, we were able to rule out several kinases, including PKC, protein kinase A, protein kinase B and extracellular-signal-regulated kinase 1 and 2 (results not shown). A different approach was to use immunoprecipitated PLD1 as an affinity matrix to enrich for a kinase activity from a tissue source. Several preliminary experiments suggested that detergent-extracted brain membranes were an appropriate source. We found that if immunoprecipitated PLD1 was mixed with such an extract, we could enrich more than 5-fold for a kinase activity that phosphorylated PLD1 (Figure 4A, compare lane 2 with lane 4). In parallel experiments, we tried to determine the size of the kinase by in-gel kinase assays. In those assays a suitable substrate is co-polymerized in a polyacrylamide gel such that after electrophoresis and renaturation, the resolved kinase phosphorylates its nearby polymerized substrate [11]. This phosphorylation event is the marker for the molecular mass of the kinase of interest. We used histone H1 as the substrate, since it was the better reporter of the differences in phosphorylation between PLD1 and S911A (see Figure 3). Using this assay, we determined that the kinase that binds and phosphorylates PLD1 has a molecular mass of approximately 40 kDa (Figure 4B). In addition, the kinase from COS-7 cells that binds PLD1 appears to be identical in size with the kinase that was enriched from brain

Figure 5 Difference in *in vitro* phosphorylation between PLD1 and S911A is due to the reduced amount of kinase that binds to S911A

After transfection and immunoprecipitation, samples containing PLD1 or S911A were subjected to in-gel kinase assays with histone H1 as the substrate, as described for Figure 4.

membrane extracts (Figure 4B, compare lanes 1 and 5 with lane 3). The increase in the amount of the kinase after incubation with the extract was very similar to the increase in the kinase activity under the same conditions (both approx. 5-fold). Using a similar assay with immunoprecipitates from COS-7 cells transfected with either wild-type PLD1 or with the S911A mutant, we found that there is 8-fold more kinase associating with PLD1 compared with S911A (Figure 5). This difference accounts very well for the difference in phosphorylation between the two proteins shown in Figures 2 and 3.

Phospho amino acid analysis of PLD1

To determine the phosphorylation sites on PLD1, we subjected preparative amounts of *in vitro* phosphorylated PLD1 to HPLC analysis of tryptic fragments coupled to MS (see the Materials and methods section). We found that phosphorylation was exclusively on serine residues, including Ser⁶¹⁰ and Ser⁶¹¹ (PLD1a numbering). These serine residues, particularly Ser⁶¹¹, are located in a site (KLFHPSSSESEQGLTR) similar in sequence to preferred phosphorylation sites for CK2 (S/TXXD/E). Based on this and the predicted size of the kinase from the in-gel kinase assays, we examined the possibility that the kinase that phosphorylates PLD1 *in vitro* is CK2.

Identification of CK2 as a serine kinase that phosphorylates PLD1

CK2 is among a few kinases that can utilize GTP in addition to ATP as a phosphate donor [15]. When PLD1 was phosphorylated *in vitro* by its associated kinase, we found that radioactive GTP was effective as a phosphate donor (Figure 6), although to a lesser extent when compared with radioactive ATP. In addition, two compounds that are known to inhibit CK2, heparin and 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB), were very effective inhibitors of the PLD1 phosphorylation *in vitro*, especially when GTP was used as the phosphate donor (Figure 6, last three lanes). To determine if an interaction of PLD1 with a GTP-utilizing kinase was evident *in situ*, we isolated Golgi-enriched membranes from CHO cell lines that express PLD1

Figure 6 Kinase described in the present study can utilize GTP and is partially inhibited by heparin and DRB

After transfection into COS-7 cells and immunoprecipitation, PLD1 on beads was subjected to a kinase assay using either $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ or $[\gamma\text{-}^{32}\text{P}]\text{GTP}$, as indicated. During the assay some samples were left untreated (—), whereas others were treated with 100 $\mu\text{g}/\text{ml}$ heparin (hep) or 10 μM DRB.

upon induction with IPTG and butyrate. Membranes from cells that had been induced overnight or left untreated were subjected to a kinase assay using radioactive GTP (Figure 7A). Following such an assay, several labelled bands were resolved, which were common to all cell lines under all conditions (Figure 7A, bottom panel). However, a band at 110 kDa became labelled only in the samples that were induced to express PLD1 (Figure 7A, bottom panel). In those samples, PLD1 was easily detectable by immunoblotting (Figure 7A, middle panel), although it was not an abundant protein, as judged by silver staining (Figure 7A, top panel). When membranes from two of the PLD1 cell lines were subjected to a kinase assay in the presence of DRB, the activity of the endogenous kinase(s) was dramatically reduced (Figure 7B). We concluded from these results that the GTP-utilizing and DRB-sensitive kinase is capable of phosphorylating PLD1 in isolated membranes.

To test directly if CK2 is the kinase in question, we performed a complementary set of immunoprecipitations and immunoblots (Figure 8). COS-7 cells transfected with PLD1 or S911A were immunoprecipitated with PLD1 antibodies and then probed with antibodies directed against the α subunit of CK2. Since we had determined that the kinase could be enriched from a membrane extract, some of the immunoprecipitates were mixed with such an extract to allow additional kinase to bind. A specific band that corresponds in size to CK2 α was present in the PLD1 immunoprecipitates, whereas it was greatly reduced in immunoprecipitates from cells expressing S911A (Figure 8A, lower right-hand panel). Preincubation of PLD1 immunoprecipitates with membrane extracts increased the amount of immunoreactive CK2 α by approx. 5-fold, in good agreement with the increase in kinase amount and activity that we saw earlier (see Figure 4). The complementary experiment was to immunoprecipitate endogenous CK2 from COS-7 cells transfected with PLD1 or S911A and determine if PLD1 was present in those samples (Figure 8B). We found that we could detect PLD1 in immunoprecipitates using CK2 α antibodies, but not in those where pre-immune serum was used (Figure 8B, upper right-hand panel).

Figure 7 Phosphorylation of PLD1 *in situ* by an endogenous kinase that utilizes GTP and is sensitive to DRB

(A) Golgi-enriched membranes were isolated from CHO cells that can express PLD1 inducibly with or without induction, as indicated. The cells included the parental clone without PLD1 (3.6), two independent clones expressing wild-type PLD1 (3.6.35 and 3.6.42) and one clone expressing a catalytically inactive PLD1 mutant (3.6.12), in which Lys⁴⁶⁶ is changed to alanine. Membranes were subjected to three treatments: (1) direct electrophoresis and silver staining (top panel); (2) electrophoresis and immunoblotting for PLD1 (middle panel); or (3) kinase assay with $[\gamma\text{-}^{32}\text{P}]\text{GTP}$, as described above, followed by electrophoresis and autoradiography (bottom panel). (B) Golgi-enriched membranes from two PLD1-expressing cell lines (3.6.35 and 3.6.42) were subjected to a kinase assay with $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ in the presence of the indicated amounts of DRB or methanol (veh), which was used to solubilize DRB. At the end of the assay, samples were analysed by SDS/PAGE and autoradiography.

Furthermore, there was a 2–3-fold difference in the amount of wild-type protein and the S911A mutant in the CK2 α immunoprecipitates, in agreement with earlier results that the kinase more effectively binds to PLD1 than S911A.

How strong is the PLD1–CK2 interaction? We compared the amount of CK2 α present in PLD1 immunoprecipitates with the amounts of ARF and PKC that were capable of interacting with PLD1 under those conditions of lysis and washing (Figure 9). Under conditions where detection of all three proteins in the lysates was very similar, only CK2 α immunoreactivity was

Figure 9 Relative strengths of the PLD1-CK2, PLD1-ARF and PLD1-PKC interactions

COS-7 cells transfected with PLD1 were lysed and immunoprecipitated (ipp) using PLD1 antibodies. Following SDS/PAGE and electrotransfer, the same blot was probed with antibodies to CK2α, PKC or ARF (Arf). The antibody concentrations used were adjusted such that the strength of the signal for the three proteins from the lysates was very similar. H, IgG heavy chains; L, IgG light chains.

apparent in the immunoprecipitated samples. Thus the PLD1-CK2 interaction is significant even when considered in comparison with the interaction of PLD1 with two of its known activators.

CK2 interacts with PLD1 through its β subunit, and phosphorylation of PLD1 requires the functional CK2 heterotetramer

CK2 is a heterotetramer of two α (or α') catalytic subunits and two regulatory β subunits. To determine which subunit is

Figure 8 Kinase described in the present study is antigenically related to CK2

(A) Immunoprecipitation with anti-PLD1. COS-7 cells transfected with plasmids encoding wild-type PLD1 or the S911A catalytically inactive point mutant were lysed and immunoprecipitated using PLD1 antibodies. After immunoprecipitation, half of the beads were mixed with a detergent extract from brain membranes (+ mem), whereas the other half were mixed with the buffer alone (- mem) for 1 h at room temperature, followed by extensive washes. The immunoprecipitated samples, as well as the lysates that were used to produce them, were resolved by SDS/PAGE, blotted, and probed first with PLD1 antibodies (top panels) followed by stripping and probing with CK2α antibodies (bottom panels). (B) Immunoprecipitation with anti-CK2α. COS cells transfected as above were lysed and immunoprecipitated with antibodies to CK2α or with pre-immune serum from the same animal. After extensive washing, the immunoprecipitated samples and the lysates were resolved by SDS/PAGE, blotted and probed first with anti-PLD1 antibodies (top panels), followed by stripping and probing with anti-CK2α antibodies (bottom panels).

Figure 10 PLD1–CK2 interaction is enhanced by the β subunit of the kinase

(A) COS-7 cells were transfected with plasmids encoding PLD1 plus CK2 α or myc-tagged CK2 β , as indicated. After lysis and immunoprecipitation (ipp) with PLD1 antibodies, the immunoprecipitated samples and the corresponding lysates were resolved by SDS/PAGE, blotted and probed with antibodies directed against PLD1, CK2 α or myc (for CK2 β), as indicated. (B) PLD1 expressed and immunoprecipitated from COS-7 cells was washed with high salt to remove endogenous bound CK2 and then incubated with bacterially expressed CK2 α (1 μ g) or CK2 α (1 μ g) plus CK2 β (0.2 μ g), as indicated, for 1 h at room temperature. Following extensive washes, the samples were resolved by SDS/PAGE, blotted and probed with antibodies to PLD1, CK2 α or CK2 β as indicated. (C) PLD1 expressed and immunoprecipitated from COS cells was washed with high salt to remove endogenous bound CK2. The resulting sample was subjected to a kinase assay using [γ -³²P]GTP in the presence of bacterially expressed CK2 α or CK2 β (0.2 μ g of purified CK2 subunit/150-mm plate of immunoprecipitated PLD1) or both, as indicated. At the end of the assay, the samples were resolved by SDS/PAGE, blotted, exposed for autoradiography (autorad; bottom panel) and probed with PLD1 antibodies (top panel).

responsible for interacting (either directly or indirectly) with PLD1, we co-transfected COS-7 cells with PLD1 and plasmids encoding either the α or the β subunit alone or both subunits together. After transfection, we immunoprecipitated the samples with PLD1 antibodies, blotted, and probed with PLD1, CK2 α or myc (for CK2 β) antibodies (Figure 10A). When CK2 α or CK2 β was co-transfected singly with PLD1, only CK2 β was enriched in the immunoprecipitates. There was no increase in the amount of CK2 α recovered, although the overexpressed protein was

Table 1 Phosphorylation of PLD1 by CK2 does not affect PLD1 catalytic activity

PLD1 activity in immunoprecipitates was measured as described in the Materials and methods section. Immunoprecipitates of PLD1 were incubated in the presence or absence of CK2 with or without Mg²⁺ ions. GTP was present in all assays. Following incubation, the immunoprecipitates were extensively washed with PBS and analysed in an exogenous substrate assay for PLD activity [14]. All assays were carried out in the absence or presence of recombinant ARF6 (250 nM) and PKC β II (70 nM). This experiment was carried out independently three times with duplicate samples; a representative set of results is shown.

PLD1 immuno-precipitation/kinase conditions	PLD1 activity (pmol of phosphatidylcholine hydrolysed/30 min)	
	– ARF6/PKC β II	+ ARF6/PKC β II
– Mg ²⁺ – CK2	0	10.3
+ Mg ²⁺ – CK2	0.6	7.0
– Mg ²⁺ + CK2	0.8	9.8
+ Mg ²⁺ + CK2	0	6.0

clearly evident in the lysate (Figure 10A, lane 1 compared with lane 2, and lane 5 compared with lane 6). However, when plasmids encoding both subunits were co-transfected with PLD1, the presence of overexpressed CK2 β resulted in additional CK2 α in the immunoprecipitates (Figure 10A, lane 6 compared with lane 8). These results suggest that CK2 β plays an important role in the formation of the CK2–PLD1 complex. A similar result was obtained when immunoprecipitated PLD1 from COS-7 cells was washed with high salt, to remove endogenous CK2, and was allowed to bind recombinant CK2 α alone or CK2 α in the presence of CK2 β (Figure 10B). Again, the presence of the β subunit enhanced the amount of CK2 α bound to PLD1. Finally, phosphorylation of PLD1 by recombinant CK2 was examined after the endogenous CK2 was removed with a high-salt wash (Figure 10C). We found that the presence of the β subunit was essential for phosphorylating PLD1. These results suggest that phosphorylation of PLD1 by CK2 depends on the formation of a functional complex between PLD1 and the entire heterotetramer of CK2.

Phosphorylation of PLD1 by CK2 does not alter PLD1 catalytic activity

We addressed the possibility that the PLD1–CK2 interaction and subsequent phosphorylation of PLD1 affect the catalytic activity of PLD1. Phosphatidylcholine hydrolysis by PLD1 was measured in the presence or absence of CK2 with or without Mg²⁺ ions, which are required for phosphorylation of PLD1 by CK2 (Table 1). Although the presence of Mg²⁺ had a small inhibitory effect on the catalytic activity of PLD1, we were unable to measure a change in catalysis attributable to the presence of CK2 or the phosphorylation of PLD1 by CK2. We conclude from this data that neither the PLD1–CK2 interaction nor the phosphorylation of PLD1 by CK2 affects the catalytic activity of PLD1.

DISCUSSION

We describe in the present study an association between PLD1 and CK2 that seems to be mediated via the regulatory β subunit of the kinase and results in phosphorylation of PLD1. Because the kinase was originally detected as a phosphorylating activity during PLD1 immunoprecipitation, the question of the specificity

of this interaction was of some concern. Several observations argue that the PLD1–CK2 association is specific. First, the kinase activity was evident only when PLD1 was immunoprecipitated using PLD1 antibodies. Second, there was a clear difference in the amount of kinase between immunoprecipitates of PLD1 and those of S911A under conditions in which both proteins were present in the immunoprecipitates at similar levels. This constitutes a very good control against non-specific effects of immunoprecipitation. Third, the same kinase could be enriched when PLD1 on beads was incubated with brain membrane extracts, indicating that the kinase could bind to PLD1 outside the cellular setting, and under fairly stringent extraction conditions [1% (v/v) detergent]. Since the association could be abolished with high salt (results not shown), the PLD1–CK2 interaction is probably reversible. Based on the relative abundance of CK2 in total lysates and its relatively low levels in the immunoprecipitates, we suggest that only a small fraction of endogenous CK2 interacts with PLD1. This is perhaps not surprising, given that CK2 is a multifunctional kinase with over 100 substrates, many of which are nuclear proteins [15,16]. Finally, the observation that immunoprecipitated PLD1 is capable of binding recombinant CK2 suggests that the PLD1–CK2 interaction may be direct. We note that the PLD1–CK2 interaction described in the present study concerns overexpressed PLD1, since we and others have found it difficult to detect endogenous PLD protein with the antibody reagents currently available.

Two observations argue that the interaction between PLD1 and CK2 *in vitro* is also relevant *in vivo*. First, there was a good correspondence of the phosphorylation levels of wild-type PLD1 and the S911A mutant *in vivo* and *in vitro* (see Figures 2 and 3), and the simple interpretation of this result is that CK2 is partially responsible for the observed phosphorylation *in vivo*. Second, Golgi-enriched membranes contained a GTP-utilizing and DRB-sensitive kinase activity capable of phosphorylating PLD1, and, based on our results, this kinase activity may be related to CK2. However, it is very likely that additional kinases interact with and phosphorylate PLD1 as part of the regulatory requirements of this enzyme. One such kinase is PKC [9].

The association between PLD1 and CK2 seems to take place on membranes. While wild-type PLD1 localizes primarily to membranes, the S911A mutant mislocalizes to the cytosol and shows a reduced interaction with CK2. Further support comes from the observation that PLD1 mutants with altered pleckstrin homology domains and/or palmitoylation sites have altered membrane localization, and a concomitant change in the amount of CK2 with which they associate (I. G. Ganley, unpublished work). Finally, the kinase activity associated with PLD1 was enriched from brain membrane extracts and was present in Golgi-enriched membranes from CHO cells. We have determined that the CHO Golgi-enriched membranes contained immunoreactive CK2 α , but, interestingly, the amount of the kinase was not altered as a function of PLD1 expression (results not shown). Thus although CK2 is capable of phosphorylating PLD1 on membranes, the available data argue against a role for PLD1 as a receptor for CK2 on membranes.

What is the significance of the PLD1–CK2 interaction? Enhancing the phosphorylation state of PLD1 by CK2 activity *in vitro* did not affect PLD catalytic activity, which argues against strong effects of the CK2–PLD1 interaction on catalysis. Two other possibilities are interesting to consider. Recent work by Exton and colleagues has suggested that serine/threonine phosphorylation of PLD1 is important for the structural stability of the protein, especially when the N- and C-terminal domains are co-expressed separately such that their interaction

reconstitutes PLD activity [17]. It is possible that CK2 is the kinase that mediates this phosphorylation, and this will be determined once the exact sites and residues that are phosphorylated in the domain reconstitution are known. An alternative possibility for the regulation of PLD1 by CK2 may involve phosphorylation-dependent trafficking of PLD1. Several proteins that traffic through the *trans*-Golgi network en route to endosomes or the plasma membrane contain putative CK2 phosphorylation sites in their cytoplasmic domains. For some of these proteins, the integrity of the CK2 site is important for correct localization [18–21]. Along similar lines, it has been recently reported that regulation of type-II α phosphatidylinositol phosphate kinase by CK2 leads to altered membrane localization of this enzyme [22]. In addition, it was recently shown that phosphorylation of the vesicle-tethering protein p115 by a CK2-like activity is relevant for Golgi reassembly *in vitro* [23]. Thus CK2 appears to be involved in several trafficking-related events, especially at the level of sorting. It is possible that PLD1 interacts with CK2 as part of a localization requirement, or it may interact with CK2 to ultimately influence the trafficking of other proteins. Since PLD1 appears to be serine phosphorylated under basal conditions in the cell, CK2 phosphorylation of PLD1 may occur during the post-translational processing of the enzyme. This phosphorylation, then, may play an important role in signalling pathways mediated by PLD1, by stabilizing and correctly localizing the enzyme to its substrate and regulators in cellular membranes.

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