Mol. Cells, Vol. 10, No. 6, pp. 669–677

Molecules and Cells

Role of Hippocalcin in Ca^{2+}-induced Activation of Phospholipase D

Jae-Keun Hyun, Changsuek Yon, Yong-Seok Kim, Dong-Young Noh[1], Kweon-Haeng Lee[2], and Joong-Soo Han*

Institute of Biomedical Science and Department of Biochemistry, College of Medicine, Hanyang University, Seoul 133-791, Korea;
[1] Department of Surgery, College of Medicine, Seoul National University, Seoul 151-742, Korea;
[2] Department of Pharmacology, College of Medicine, The Catholic University of Korea, Seoul 137-701, Korea.

(Received on July 5, 2000)

The role of hippocalcin as a novel mediator in the PKC-independent Ca^{2+}-induced phospholipase D (PLD) activation pathway was investigated. Hippocalcin was expressed in the Sf9 insect cell expression system because the myristoylation of this protein is essential for its function. PLD and Cdc42 proteins were prepared from a rat brain cell membrane and cytosol, respectively. The recombinant hippocalcin was expressed in the Sf9 cell using expression vector pVL1393. The hippocalcin expressed was purified as a single band on PAGE following the hydrophobic phenyl HPLC and TSKgel G3000SW gel filtration HPLC. The molecular size of the rat brain hippocalcin expressed in this system was estimated to be 22 kDa. Myristoylated hippocalcin migrated faster than the non-myristoylated form on SDS-PAGE. Less than 10% of the total hippocalcin expressed was myristoylated in this baculovirus expression system. PLD was extracted from rat brain membranes and chromatographically enriched 70-fold. From the rat brain cytosol, Cdc42 was purified to near homogeneity. While hippocalcin alone did not activate PLD, it increased PLD activity activated with Cdc42 1.8-fold in the presence of calcium (300 nM free calcium). In the absence of calcium in the reaction mixture, the effect of hippocalcin to facilitate Cdc42-activated PLD activity was abolished. This result suggests that hippocalcin might be one of the regulatory proteins in the PKC-independent Ca^{2+}-mediated PLD activation pathway in conjunction with the Cdc42 protein.

Keywords: Calcium; Cdc42; Hippocalcin; Phospholipase D; PKC; Rat Brain; Sf9 Cell.

* To whom correspondence should be addressed.
Tel: 82-2-2290-0623; Fax: 82-2-2294-6270
E-mail: jshan@email.hanyang.ac.kr

Introduction

Several different pathways, where small molecular G proteins, Ca^{2+}, unsaturated fatty acids, protein kinase C (PKC), protein–tyrosine kinase, and others are related, mediate the activation of phospholipase D (PLD, Billah and Anthes, 1990; Chalifa *et al*., 1990; Cockcroft, 1992; Cockcroft *et al*., 1994; Conricode *et al*., 1992; Exton, 1994; Gustavsson *et al*., 1994; Inoue *et al*., 1995; Kiss and Anderson, 1994; Massenburg *et al*., 1994; Mohn *et al*., 1992; Uings *et al*., 1992). Recently, many experimental results regarding PKC-dependent and small-G-protein-dependent PLD activation pathways have been reported. ADP-ribosylation factor (Arf) is one of the G proteins that activate PLD, and phosphatidylinositol 4,5-bisphosphate (PIP_2) has been known to be an essential cofactor for the activation of PLD (Brown *et al*., 1993; 1995; Cockcroft *et al*., 1994; Liscovitch *et al*., 1994; Pertile *et al*., 1995). Besides Arf, other G proteins such as Rho family proteins (RhoA, Cdc42 and Rac1), are also related in the activation of PLD (Bowman *et al*., 1993; Malcolm *et al*., 1994; Shin, *et al*., 1999; Siddiqi *et al*., 1995; Singer *et al*., 1995).

An increase of the intracellular Ca^{2+} level was reported to mediate the activation of PLD via activation of PKC (Conricode *et al*., 1994; Geny and Cockcroft, 1992), and this has generally been accepted as true. Gustavsson *et al*. (1994) suggested PKC-independent Ca^{2+} mediated the PLD activation pathway by showing that PLD activation was observed with an increased intracellular Ca^{2+} level when PKC activity was completely downregulated.

Han *et al*. (1998) isolated the 22 kDa protein from rat brain cytosol that was involved in the activation of PLD and identified the protein as Ca^{2+}-binding hippocalcin using peptide sequencing. Hippocalcin is a specific protein expressed only in the pyramidal nerve

cell of the hippocampus that has three EF-hand structural Ca^{2+}-binding domains and a myristoylation site in the amino terminal (Kobayashi *et al.*, 1993). When the intracellular Ca^{2+} level is increased, hippocalcin is translocated to the cell membrane after binding with Ca^{2+} (Kawamura *et al.*, 1992; Polans *et al.*, 1991; Takamatsu *et al.*, 1992). Myristoylation of the protein is essential for the translocation to the membrane (Dizhoor *et al.*, 1992; Zozulya and Stryer, 1992).

On the basis of these results, we tried to determine the role of hippocalcin after binding with Ca^{2+} in the PKC-independent Ca^{2+}-induced PLD activation pathway in conjunction with the Cdc42 protein.

Materials and Methods

Expression of hippocalcin cDNA with baculovirus

Subcloning of hippocalcin cDNA Recombinant hippocalcin cDNA in the TA cloning vector (PCR3.1 vector) was subcloned in the baculovirus transfer vector pVL1393. Hippocalcin cDNA cloned in TA cloning plasmid (PCR3.1 plasmid) and pVL1393 vector were double-digested with BamH1 and EcoR1 restriction endonucleases and isolated with the Wizard™ DNA Clean-Up System (Promega) after electrophoresis in low-melting agarose gel. Hippocalcin cDNA (120 ng) and pVL1393 vector DNA (200 ng) were ligated for 16 h in 10 μl of ligation buffer including 0.2 units of T4 ligase at 16°C to make recombinant pVL1393 vector (pVL1393-HP610). Recombinant vector pVL1393-HP610 was tranformed to competent *E. coli* JM109, and 200 μl of the transformed mixture was spread on an LB agar plate including 100 μl of ampicillin: then it was incubated for 12 h at 37°C. The transformation was confirmed by colony PCR using F-152-BamH1 primer and R-761-EcoR1 primer.

Baculovirus expression of recombinant pVL1393 vector (pVL1393-HP610) A BaculoGold™ transfection kit (Pharmingen) was used to express rat brain hippocalcin cDNA in insect cell Sf9. Sf9 host cells (3×10^6 cells) were incubated for 15 min at 27°C for attachment to a 60-mm culture dish. The cell culture media were removed, and 1 ml of BaculoGold™ transfection buffer A was added to the culture dish. BaculoGold™ virus DNA (0.5 μg) was mixed with 2.0 μg of recombinant vector pVL1393-HP610 and incubated for 5 min at room temperature. After incubation, 1 ml of BaculoGold™ transfection buffer B was added. The reaction mixture was added to the culture dish including BaculoGold™ transfection buffer A. The transfection buffer was removed after 4 h of incubation at 27°C, and 3 ml of TNM-FH culture medium enriched with 10% of bovine serum was added. Cells were collected and centrifuged for 20 min at $1{,}000 \times g$ to obtain supernatant after 4 d of incubation at 27°C. For the first amplification of the recombinant virus particles, 200 μl of the supernatant was added to a 100-mm culture dish preattached with 7×10^6 Sf9 cells and incubated for 3 d at 27°C. The cells were collected and centrifuged for 20 min at $1{,}000 \times g$ and the supernatant was transferred to a fresh tube. For the second amplification of the recombinant virus particles, 50 μl of the supernatant from the first amplification was inoculated to 2×10^7 Sf9 cells attached on a 150-mm culture dish and incubated for 3 d at 27°C. The cells were collected and centrifuged for 10 min at $1{,}000 \times g$ and the supernatant was kept at –70°C in a deep freezer until used. The inoculated cells were washed with PBS and were used to determine the level of hippocalcin expression. For the detection of myristoylated hippocalcin, 1 μCi/ml of [^{3}H]-myristic acid (DuPont NEM) was added to the media in the second amplification step.

Isolation and purification of the expressed hippocalcin pVL1393-HP610 transfected Sf9 cells were washed with chilled PBS, ruptured with sonication, and centrifuged for 30 min at $10{,}000 \times g$ to obtain a cytosol fraction. Hippocalcin expression was confirmed with 12.5% SDS–PAGE. Ammonium sulfate was added to 1.2 M and centrifuged for 30 min at $10{,}000 \times g$. The supernatant was loaded on a Phenyl-5PW column (7.5 × 75 mm, TosoHaas). The mobile phase was composed of 20 mM Tris (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 5% ethylene glycol, and 1.2 M ammonium sulfate. The flow rate was 0.2 ml/min and the fractionation time was 4 min for each tube. Linear gradients for ammonium sulfate and ethylene glycol were applied from the initial time to 180 min with a concentration of 1.2–0 M and 5–75%, respectively. Fractions from numbers 30 to 33 were pooled and concentrated to a volume of 2 ml with ultrafiltration (Centricon-10, Amicon). The concentrate was loaded on a TSKgel G3000SW gel filtration column (7.5 × 300 mm, TosoHaas). The mobile phase was composed of 20 mM Tris (pH 7.5), 1 mM DTT, 1 mM EDTA, and 0.3 M NaCl. The flow rate was 0.5 ml/min and the fractionation volume was 1 ml. For detection of myristoylated hippocalcin, pVL1393-HP610 transfected Sf9 cells were cultured in culture media containing 1 μCi/ml of [^{3}H]-myristic acid. Isotope-labeled hippocalcin was isolated with the same method and separated on 18% SDS–PAGE. Labeled hippocalcin was transferred to a nitrocellulose membrane and autoradiographed for 3 d at –70°C in a deep freezer.

Purification of PLD from rat brain cell membrane

Isolation of rat brain cell membrane All the experiments were performed at 4°C, except for those specified otherwise. Four hundred grams of rat brain was dissolved in 4 l of buffer A (20 mM HEPES, pH 7.0; 1 mM EGTA; 0.1 mM DTT; 1.5 mM phenylmethylsulfonyl fluoride; 1.5 μg/ml of leupeptin; and 1.5 μg/ml of aprotinin) and homogenized. The mixture was centrifuged for 20 min at $1{,}000 \times g$ and the supernatant was transferred to a fresh tube. A pellet was redissolved in 2 l of buffer A and homogenized again. The mixture was centrifuged and the supernatants were pooled. The supernatant pooled was ultracentrifuged for 1 h at $100{,}000 \times g$, and the pellet containing the cell membrane was recovered to isolate PLD.

Purification of PLD from the cell membrane The pellet was stirred for 1 h in 1.2 l of buffer A enriched with 1.0% Triton-X100 and 0.3 M NaCl and was ultracentrifuged for 1 h at

100,000 × *g* to isolate membrane proteins. The protein extract solution (about 8 g of total protein) was loaded on a Heparin-Sepharose CL-6B column (5 × 30 cm) equilibrated with buffer B (20 mM HEPES, pH 7.0; 1 mM EGTA; 0.1 mM DTT; and 0.1% Triton-X100) containing 0.3 M NaCl. The column was washed with 600 ml of buffer B and eluted with an NaCl gradient of 0.3–1.0 M. The fractionation volume was 20 ml for each tube and was monitored by measuring the PLD activity with the existence of Arf isolated from rat brain and GTPγS. Fractions with PLD activity were pooled and loaded on a CM-5PW cation-exchange column (21.5 × 150 cm) equilibrated with buffer B. The sample volume was 300 ml, and the total protein was 600 mg. To minimize the effect of detergent on PLD activity, Triton-X100 was substituted with *n*-octyl-D-glucopyranoside. For this, the column was washed with 500 ml of buffer C (20 mM HEPES, pH 7.0; 1 mM EGTA; 0.1 mM DTT; and 0.7% *n*-octyl-D-glucopyranoside). The flow rate was 5 ml/min. Elution was made by buffer C with an NaCl gradient of 0–1.0 M within 40 min and with 1.0 M of NaCl for another 20 min. Each fraction was measured for PLD activity. The fractionation volume was 5 ml for each tube. Fractions with PLD activity were pooled and dialyzed against buffer D (20 mM HEPES, pH 7.0; 1 mM EGTA; 0.1 mM DTT; 0.7% *n*-octyl-D-glucopyranoside; and 2.5 μg/ml of aprotinin). The sample was centrifuged and the supernatant (25.8 mg of total protein) was loaded on a Mono Q HR 5/5 column (Pharmacia Biotech) equilibrated with buffer D and eluted with an NaCl gradient of 0–1.0 M for under 40 min. The fractionation volume was 1 ml, and fractions with PLD activity were pooled. The sample was loaded on a Mono S HR 5/5 column (Pharmacia Biotech) equilibrated with buffer C. Proteins were eluted with the same condition as Mono Q HR 5/5 column chromatography. Fractions with PLD activity were pooled and applied to Heparin-5PW column (7.5 × 75 mm) chromatography. The flow rate was 1 ml/min and the NaCl gradient was 0–0.5 M within 15 min and 0.5–1.0 M in another 45 min, PLD fractions were pooled (12 ml of volume and 700 μg of protein) and were kept at –80°C in a 50 μl aliquot until used.

Purification of Cdc42 from rat brain cell cytosol

DEAE-Sephacel anion column chromatography Rat brain cell cytosol fractionated with 35–70% of ammonium sulfate was dissolved in 400 ml of buffer E (10 mM Tris, pH 8.0; 1 mM EDTA; 1 mM DTT; 1 μg/ml of leupeptin and aprotinin, respectively) and dialyzed against the same buffer. The sample was centrifuged and the supernatant (400 ml of volume and 10 g of total protein) was loaded on a DEAE-Sephacel column (5 × 20 cm). The column was washed with 800 ml of buffer E and eluted with 2 l of buffer E with an NaCl gradient of 0–0.5 M. Fractions exhibiting PLD activation activity (fractions 80–90) were collected and pooled.

DEAE-5PW anion-exchange-column chromatography The sample was dialyzed against buffer E and loaded on a DEAE-5PW column (21.5 × 150 mm) and eluted with buffer E. The NaCl gradient was 0–0.3 M within 80 min The flow rate was 5 ml/min, and the fractionation time was 1 min for each tube. The peak fractions (fractions 23–28) were pooled.

Hydroxyapatite HPLC The sample was dialyzed against buffer H (20 mM Tris, pH 7.5; 5 mM $MgCl_2$; 1 mM DTT; 5% ethylene glycol; and 0.1 M KCl) and applied to HA-1,000 column (7.5 × 75 mm, TosoHaas) chromatography. The flow rate was 1 ml/min with a KPO_4 gradient of 0–0.1 M within 80 min, and the fractionation volume was 1 ml for each tube.

Phenyl-5PW hydrophobic chromatography Ammonium sulfate was added to a concentration of 1.2 M and centrifuged to remove the pellet. The supernatant was loaded on a Phenyl-5PW column (7.5 × 75 mm) equilibrated with buffer I (20 mM Tris, pH 7.5; 5 mM $MgCl_2$; 1 mM DTT; 5% ethylene glycol; and 1 mM EDTA) containing 1.2 M ammonium sulfate. Elution was made with a 0.2-ml/min flow rate of buffer I and 1.2–0 M ammonium sulfate gradient and 5–75% ethylene glycol gradient within 180 min. The fractionation time was 4 min for each tube (Fig. 4A).

Measurement of PLD activity The method proposed by Han *et al.* (1998) was modified and adopted for the experiments. The mixed lipid vesicle was made with PE, PIP_2, and PC (molar ratio of 16:1.4:1) and [choline-methyl-^{3}H](pam)$_2$PC was added to 20,000 cpm. The reaction buffer was composed of 50 mM HEPES (pH 7.5), 3 mM EGTA, 80 mM KCl, 2,5 mM $MgCl_2$, 2 mM $CaCl_2$ and 5 μM GTPγS. The hippocalcin effect on the PLD activity activated by Cdc42 was determined in the reaction buffer containing 40 nM of Cdc42. The hippocalcin effect on the PLD activity with various levels of intracellular calcium concentration (0–50 mM) was also measured. PLD (1–5 μl) was added to the reaction mixture and incubated for 30 min at 37°C. The reaction was stopped by adding 1 ml of stop solution ($CHCl_3$:CH_3OH:conc. HCl = 50:50:0.3, v/v) and 0.35 ml of 1 M HCl solution containing 5 mM EGTA. The reaction mixture was centrifuged, and 0.5 ml of supernatant was recovered. The PLD activity was measured by counting [^{3}H]-choline with a liquid scintillation counter.

Western blot analysis Proteins in column fractions were resolved by electrophoresis through 12% SDS–polyacrylamide gels and transferred to a nitrocellulose sheet (BA83, Schleicher & Schuell, Keene, NH). After incubations with primary antibody (1 μg/ml) and alkaline phosphatase-conjugated secondary antibody (KPL, Gaithersburg, MD), immunoreactive proteins were visualized by an alkaline phosphatase detection kit (BCIP/NBT, KPL).

Binding of [^{35}S]GTPγS after electrophoretic transfer to nitrocellulose Binding of [^{35}S]GTPγS was performed using a method described previously (Sommer and Song, 1994). Briefly, following electrophoresis on 12% SDS–polyacrylamide gel, proteins were transferred to a nitrocellulose sheet and incubated for 1.5 h at room temperature in 100 ml of 50 mM Tris–HCl (pH 7.5) containing 0.1% BSA, 5 mM $MgCl_2$, 2 mM DTT, and 0.1% (v/v) Triton X-100. The solution was then replaced with 10 ml of the same buffer containing 2.7 nM [^{35}S]GTPγS. After incubation at room temperature for 1.5 h, the sheet was washed six times with 100 ml of the same buffer for 15 min, dried, and autoradio-

graphed with Kodak X-Omat film with an intensifying screen.

Results

Expression of rat brain hippocalcin with baculovirus expression vector pVL1393 Rat brain hippocalcin cDNA of 610-bp size was isolated from PCR3.1-HP610 and subcloned between the BamH1 and EcoR1 sites of pVL1393, and the recombinant plasmid was named pVL1393-HP610. The recombinant plasmid was transfected in the moth uterus cell Sf9. Cytosol was isolated from the cultured Sf9 cells and applied to 12.5% SDS-PAGE. Figure 1 shows the expression of hippocalcin by the 22 kDa band only in transfected cells.

Isolation and purification of the expressed hippocalcin Ethylene glycol gradient HPLC was applied to fractionate the transfected Sf9 cell cytosol. Each fraction was checked for 22 kDa hippocalcin with 12.5% SDS-PAGE and silver stain. Most of the hippocalcin was eluted in fraction numbers 30–33 (Fig. 2A). The fractions were pooled and applied to TSKgel G3000SW gel filtration chromatography to isolate pure hippocalcin (Fig. 2B). A purity check with 18% SDS-PAGE showed over 95% purity. Figure 3A shows a 20 kDa small band beside the 22 kDa main band, and the small band was assumed to be myristoylated hippocalcin because ARF showed faster migration on SDS-PAGE when myristoylated (Randazzo *et al.*, 1995). To confirm whether the 20 kDa protein is myristoylated hippocalcin, the transfected Sf9 cells were labeled with [^{3}H]-myristic acid. The purified hippocalcin was electrophoresed on 18% SDS-PAGE and transferred to a nitrocellulose membrane. The autoradiographic result showed that the 20 kDa band is the myristoylated one on SDS-PAGE (Fig. 3B). This result indicates that the 22 kDa band is nonmyristoylated hippocalcin, while the 20 kDa band is myristoylated hippocalcin. In this experiment, less than 10% of the expressed hippocalcin is myristoylated only.

Partial purification of rat brain cell membrane PLD Arf-dependent and PIP_2-requiring rat brain cell membrane PLD was purified 70-fold with sequential chromatography (Heparin-Sepharose CL-6B column, CM-5PW

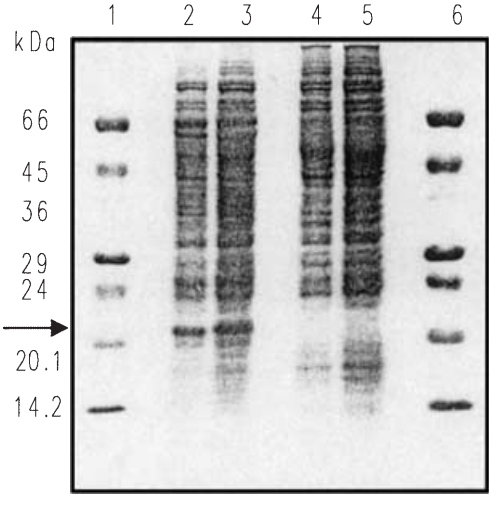

Fig. 1. SDS-PAGE of cytosolic proteins from normal and transfected Sf9 cell with pVL1393–HP610. Samples were subjected to 12.5% SDS-PAGE and stained with Coomassie blue. Lanes 1 and 6, standard SDS-PAGE protein markers; lane 2, 20 μg of cytosol from transfected Sf9 cell with pVL1393-HP610; lane 3, 40 μg of cytosol from transfected Sf9 cell with pVL1393-HP610; lane 4, 20 μg of cytosol from normal Sf9 cell; lane 5, 40 μg of cytosol from normal Sf9 cell. The arrow indicate expressed hippocalcin in transfected Sf9 cell with pVL1393-HP610.

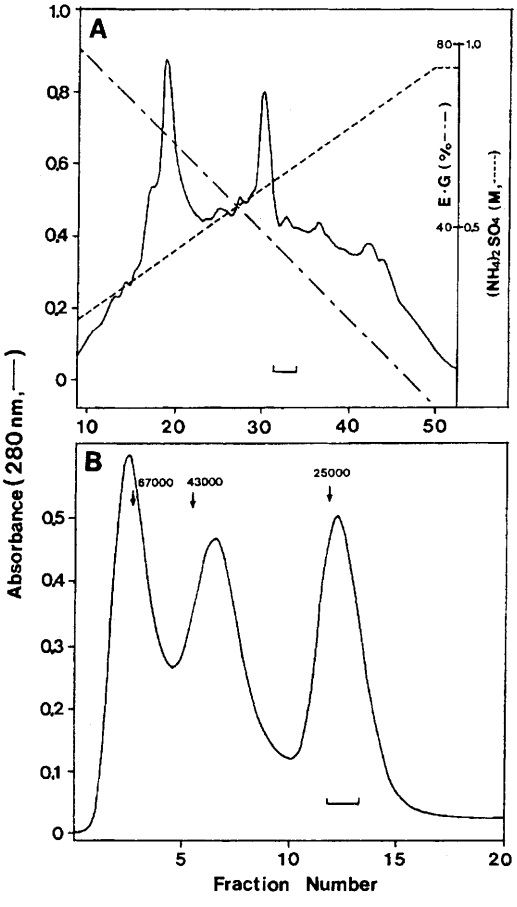

Fig. 2. Purification of hippocalcin from cytosol of transfected Sf9 cell with pVL1393-HP610. **A.** Cytosol was subjected to hydrophobic chromatography on a Phenyl-5PW HPLC column. The bar under the elution profile indicates fractions that contain hippocalcin. **B.** Hippocalcin fraction pooled from the Phenyl-5PW HPLC step was applied to TSKgel G3000SW gel filtration HPLC.

preparative column, Mono Q and S column, and Heparin-5PW analytical column, Table 1). To minimize the effect of surfactant on the activity of PLD, Triton X-100 was substituted with *n*-octyl-D-glucopyranoside in the CD-5PW preparative HPLC step. Partially purified PLD showed substrate specificity for PC but not for phosphatidylethanolamine or phsophatidylserine (Table 2).

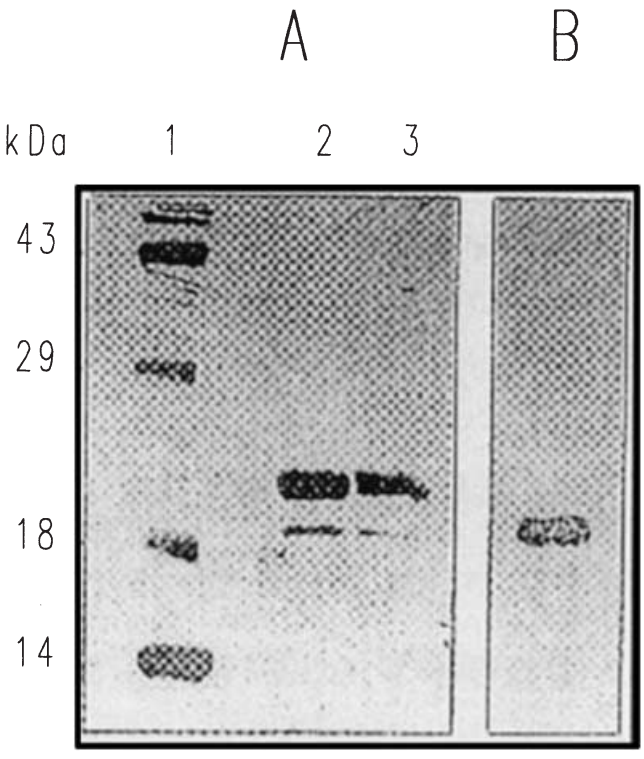

Fig. 3. Electrophoretic analysis of purified hippocalcin in fractions of gel filtration HPLC on 18% SDS-PAGE. **A.** Approximately 2 μg of a gel filtration fraction was loaded on each lane of the gel and stained with Coomasie blue. Lane 1, standard SDS-PAGE protein marker; lane 2, fraction number 13 of gel filtration chromatography shown in Fig. 2B; lane 3, fraction number 14 of gel filtration chromatography shown in Fig. 2B. **B.** Culturing and labeling with [^{3}H]-myristic acid were performed as described in **Materials and Methods**. After electrophoresis of purified hippocalcin, proteins were transferred to a nitrocellulose membrane and developed for autoradiography. Only the 20 kDa region of the autoradiogram is shown in **B** as no other band was detected elsewhere on the gel.

Cdc42 protein purification from rat brain cell membrane The 35–70% ammonium sulfate precipitating fraction of rat brain cytosol was applied to DEAE-Sepharose column chromatography. Cdc42 was purified by Hydroxyapatite HPLC and ethylene glycol gradient Phenyl-5PW HPLC from fractions that contained G proteins supposed to activate PLD activities. SDS-PAGE (12%) and silver staining showed Cdc42 was eluted between fraction numbers 44 and 50, and the purity was over 80% (Figs. 4A and 4B). Also, changes in the amount of Cdc42 among fractions were parallel to the degree of GTPγS binding and immunoblot against Cdc42 (Fig. 4C).

Effect of hippocalcin on Cdc42 mediated activation of PLD The effect of hippocalcin on PLD activity activated by Cdc42 under reconstitution assay conditions of PLD (10 mM Ca^{2+}, free calcium concentration: 300 nM) showed that changes of hippocalcin concentration did not affect the activity of PLD alone but that it increased the activity of PLD activated with Cdc42 by 1.8 times at a of concentration of 160 nM (Fig. 5). Hippocalcin showed a maximum effect on PLD activity with the presence of 10 mM Ca^{2+}, but it showed no effect when Ca^{2+} was removed from the reaction mixture (Fig. 6).

Discussion

When we tried to purify the Cdc42 protein from rat brain cytosol, the Cdc42 concentration in each fraction was not consistent with the PLD activation profile, showing that the PLD activation activity peak had shifted (Han *et al.*, 1998). The shifted PLD activation activity peak fractions contained unknown 20 kDa protein, which turned out to be hippocalcin after peptide sequencing. We found that purified Cdc42 activated PLD in a Ca^{2+}-dependent

Table 1. Purification of PLD from rat brain.

Purification step	Volume (ml)	Protein (mg)	Total activity (nmole/ min)	Specific activity (nmole/min/mg)	Yield (%)	Purification fold
Triton X-100 extract	1,200	8,000				
Heparin-sepharose	300	600	562.8	0.86	100	1.0
CM-5PW preparative	60	25.8	191.5	7.40	34	8.6
Mono Q	20	8.0	67.4	8.43	12	9.8
Mono S	12	2.1	46.2	22.0	8	25.6
Heparin-5PW analytical	12	0.7	42.2	60.6	7.5	70.4

Pools from fractionation steps were assayed for PLD activity as indicated in the presence of Arf (0.1 μM final concentration) from rat brain cytosol described in **Materials and Methods**. Assays were conducted at 37°C for 30 min in the presence of 5 μM GTPγS.

manner in the presence of this protein. This observation is very meaningful to us, because there is a report showing that an increased Ca^{2+} level in a cell resulted in PLD activation even in complete PKC downregulation (Gustavsson *et al.*, 1994). Therefore, we want to postulate a theory that hippocalcin may be involved in Ca^{2+}-induced activation of PLD.

Hippocalcin is a Ca^{2+}-binding protein which is a member of the recoverin family and was first identified by Kobayashi *et al.* (1992). Recoverin family proteins are Ca^{2+}-binding proteins found in the retina (Dizhoor *et al.*, 1991; Kawanura and Murakami, 1991; Kutuzov *et al.*, 1991; McGinnis *et al.*, 1992) and the brain (Kobayashi *et al.*, 1992; 1993; Kuno *et al.*, 1992; Takamatsu *et al.*, 1992; Terasawa *et al.*, 1992). Retinal recoverin proteins are known to be involved in the regulation of signaling by light and to activate guanylate cyclase after binding with Ca^{2+} or to maintain the cyclic GMP-phosphodiesterase activity which is activated by light (Dizhoor *et al.*, 1991; Kawamura and Murakami, 1991). The physiological activities of brain recoverin proteins including hippocalcin are not well known. Brain recoverin proteins are supposed to be involved in Ca^{2+}-related brain signaling owing to the fact that the structures of these proteins are similar to those of retinal recoverin proteins. Hippocalcin is a specific protein expressed only in the pyramidal nerve cell of the hippocampus which has three EF-hand structured Ca^{2+}-binding domains and a myristoylation site in the amino terminal (Kobayashi *et al.*, 1993). This is a common characteristic of recoverin proteins. Most of the myristoylated proteins are involved in signal transduction in cells, because myristoylation is very important in the interaction of proteins with cell membranes (James and Olson, 1990; Towler *et al.*, 1988). Recoverin proteins bind Ca^{2+} before translocation to a membrane (Kawamura *et al.*, 1992; Polans *et al.*, 1991; Takamatsu *et al.*, 1992), and myristoylation is essential in the translocation of proteins to the membrane

Table 2. Substrate specificities of rat brain Arf-dependent PLD.

Lipid	Relative PLD activity (%)
Phosphatidylcholine (PC)	100
Phosphatidylethanolamine (PE)	ND (2)
Phosphatidylserine (PS)	ND (0.4)

ND, not detectable.
Arf-dependent PLD was assayed as described in **Materials and Methods**. The activity of Arf-dependent PLD for PC hydrolysis was 60 nmol/mg/min. The total amount of PC was 0.51 nmol per assay. The same amount of PS was replaced with PC in mixed lipid vesicles (PE/PIP_2/PS, molar ratio 16:1.4:1). In the case of PE, PE/ PIP_2 mixed lipid vesicles were used (molar ratio 16:1, amount of PE per assay was 8.16 nmol).

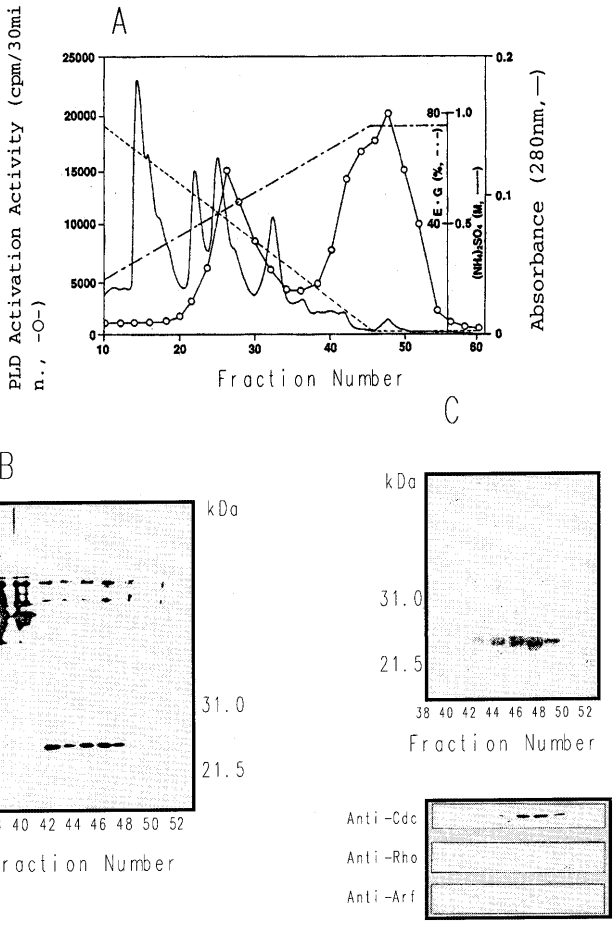

Fig. 4. Chromatography of Cdc42 with Phenyl-5PW analytical HPLC column. **A.** Peak fraction pooled from the HA-1000 HPLC step was fractionated through a TSKgel Phenyl-5PW analytical HPLC column and assayed as described in **Materials and Methods**. **B.** An aliquot (20 μl) of every second fraction was diluted with an equal volume of Laemmli SDS sample buffer, and 20 μl of this was subjected to electrophoresis using 12% PAGE. The proteins were visualized by silver staining. **C.** [^{35}S]GTPγS binding to the small-molecular-weight GTP binding proteins after transfer to a nitrocellulose membrane. Electrophoresis was performed as described above. Proteins were transferred to a nitrocellulose membrane, incubated in the presence of [^{35}S]GTPγS, and autoradiographed as described in **Materials and Methods**. After transfer to the nitrocellulose membrane, proteins were also blotted using antibodies specific for Cdc42Hs, RhoA or Arf.

(Dizhoor *et al.*, 1992; Zozulya and Stryer, 1992). Hippocalcin is myristoylated in amino terminal glycine residue and binds with Ca^{2+} in a submicromolar concentration of Ca^{2+}. Kobayashi *et al.* (1993) showed myristoylation was involved in the translocation of hippocalcin to a membrane after binding with Ca^{2+}.

This study was designed to explore the effect of hippocalcin on the activation of PLD by Cdc42 in the increased Ca^{2+} condition. Hippocalcin cDNA was cloned from the rat brain cDNA library, and expression

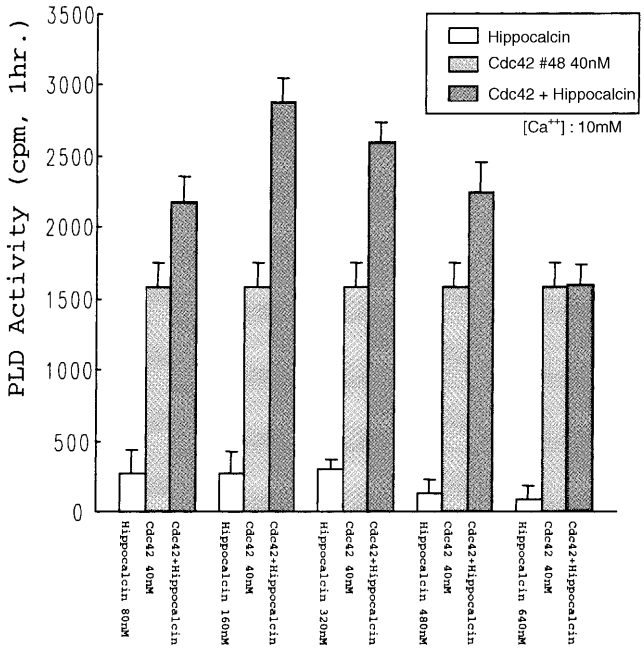

Fig. 5. Effect of hippocalcin and Cdc42 on PLD Activity. The PLD activity was determined as described in **Materials and Methods**. The reaction mixture contained 0.2 μg of PLD, 5 μM GTPγS, 40 nM Cdc42 protein, 10 mM Ca^{2+} (300 nM free Ca^{2+}), and indicated amounts of recombinant hippocalcin. Data are mean ± SD from three experiments.

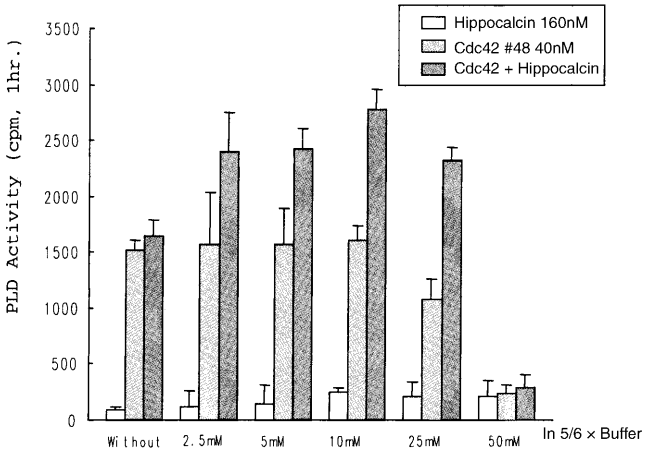

Fig. 6. Effect of calcium concentration on PLD activation activity by Cdc42 protein in the presence of hippocalcin. The PLD activity was determined as described in **Materials and Methods**. The reaction mixture contained 0.2 μg of PLD, 5 μM GTPγS, 40 nM Cdc42 protein, 160 nM recombinant hippocalcin, and indicated concentrations of calcium. Each data point represents the mean ± SD from three experiments.

and isolation of this protein were performed using baculoexpression. Rat brain hippocalcin cDNA was subcloned between the BamH1 and EcoR1 sites in MCS (multicloning site) of pVL1393 and was named pVL1393HP610. The recombinant plasmid was transfected in Sf9 cells and the protein expressed was identified on SDS-PAGE. Expression with the baculosystem showed a high yield of protein expression of transfected cDNA. The 22 kDa cytosolic protein which was not present in untransfected Sf9 cells was easily found on SDS-PAGE and was identified as hippocalcin using Northern blotting (data not shown).

Cytosolic hippocalcin was isolated with a purity of over 95% with hydrophobic Phenyl–5PW and gel filtration HPLC. SDS-PAGE showed a 20 kDa protein next to the 22 kDa hippocalcin. Myristoylation of hippocalcin as posttranscriptional modification was identified by incubating the Sf9 cells in [^{3}H]-myristic acid containing media. Hippocalcin was isolated from [^{3}H]-labeled cells and was separated on SDS-PAGE. The 20 kDa protein was identified as myristoylated hippocalcin using autoradiography of the SDS-PAGE. This result meant myristoylated hippocalcin showed faster migration on SDS-PAGE in agreement with Randazzo *et al.* (1995), who showed that the small G protein Arf showed faster migration on SDS-PAGE when myristoylated. In this study, less than 10% of the expressed hippocalcin was myristoylated. A specially designed culture condition such as myristic acid feeding is required to increase myristoylated hippocalcin in this expression system.

An increase of the hippocalcin concentration did not affect the activity of PLD alone, but it increased the activity of PLD activated with Cdc42 1.8-fold at a concentration of 160 nM. When Ca^{2+} was removed from the reaction mixture, the effect of hippocalcin against PLD activation vanished. Hippocalcin showed the maximum effect against PLD activation with the presence of 10 mM Ca^{2+} (free Ca^{2+} concentration of 300 nM). At the optimal Ca^{2+} concentration, hippocalcin showed the maximal effect on Cdc42 mediated PLD activation at a concentration of 160 nM. When the concentrations of Ca^{2+} and hippocalcin exceeded 10 mM and 160 nM, respectively, PLD activity decreased. This might be due to the detection method for PLD activity adopted reconstitution assay using a mixed phospholipid vesicle, because the mixed vesicles became unstable in the high concentration of the divalent cation Ca^{2+}.

Taken together, these results indicate that hippocalcin as a Ca^{2+}-binding protein is involved in the PKC-independent Ca^{2+}-mediated PLD activation pathway as one of the regulatory proteins for PLD activity. We can suggest the role of hippocalcin in PLD activation for the first time. When the intracellular Ca^{2+} level increases, cytosolic hippocalcin binds to Ca^{2+} and translocates to the membrane and potentiates the activation of PLD activated by the Cdc42 protein.

Acknowledgments This work was supported by grant no. 2000-2-20900-003-3 from the Basic Research Program of the Korea Science and Engineering Foundation.

References

Billah, M. M. and Anthes, J. C. (1990) The regulation and cellular functions of phosphatidylcholine hydrolysis. *Biochem. J.* **269**, 281–291.

Bowman, E. P., Uhlinger, D. J., and Lambeth, J. D. (1993) Neutrophil phospholipase D is activated by a membrane-associated Rho family small molecular weight GTP-binding protein. *J. Biol. Chem.* **268**, 21509–21512.

Brown, H. A., Gutowski, S., Moomaw, C. R., Slaughter, C., and Sternweise, P. C. (1993) ADP-ribosylation factor, a small GTP-dependent regulatory protein, stimulates phospholipase D activity. *Cell* **75**, 1137–1144.

Brown, H. A., Gutowski, S., Kahn, R. A., and Sternweis, P. C. (1995) Partial purification and characterization of Arf-sensitive phospholipase D from porcine brain. *J. Biol. Chem.* **270**, 14935–14943.

Chalifa, V., Mohn, H., and Liscovitch, M. (1990) A neutral phospholipase D activity from rat brain synaptic plasma membranes. Identification and partial characterization. *J. Biol. Chem.* **265**, 17512–17519.

Cockcroft, S. (1992) G-protein-regulated phospholipases C, D and A2-mediated signalling in neutrophils. *Biochem. Biophys. Acta* **1113**, 135–160.

Cockcroft, S., Thomas, G. M. H., Fensome, A., Geny, B., Cunningham, E., Gout, I., Hiles, I., Totty, N. F., Truong, O., and Hsuan, J. J. (1994) Phospholipase D: a downstream effector of ARF in granulocytes. *Science* **263**, 523–526.

Conricode, K. M., Brewer, K. A., and Exton, J. H. (1992) Activation of phospholipase D by protein kinase C. Evidence for a phosphorylation-independent mechanism. *J. Biol. Chem.* **267**, 7199–7202.

Conricode, K. M., Smith, J. L., Burns, D. J., and Exton, J. H. (1994) Phospholipase D activation in fibroblast membranes by the alpha and beta isoforms of protein kinase C. *FEBS Lett.* **342**, 149–153.

Dizhoor, A. M., Ericsson, L. H., Johnson, R. S., Kumar, S., Olshevskaya, E., Zozulya, S., Neubert, T. A., Stryer, L., Hurley, J. B., and Walsh, K. A. (1992) The NH_2 terminus of retinal recoverin is acylated by a small family of fatty acids. *J. Biol. Chem.* **267**, 16033–16036.

Dizhoor, A. M., Ray, S., Kumar, S., Niemi, G., Spencer, M., Brolley, D., Walsh, K. A., Philipov, P. P., Hurley, J. B., and Stryer, L. (1991) Recoverin: a calcium sensitive activator of retinal rod guanylate cyclase. *Science* **251**, 915–918.

Exton, J. H. (1994) Phosphatidylcholine breakdown and signal transduction. *Biochem. Biophys. Acta* **1212**. 26–42.

Geny, B. and Cockcroft, S. (1992) Synergistic activation of phospholipase D by protein kinase C- and G-protein-mediated pathways in streptolysin O-permeabilized HL60 cells. *Biochem. J.* **284**, 531–538.

Gustavsson, L., Moehren, G., Torres-Marquez, M. E., Benistant, C., Rubin, R., and Hoek, J. B. (1994) The role of cytosolic Ca2+, protein kinase C, and protein kinase A in hormonal stimulation of phospholipase D in rat hepatocytes. *J. Biol. Chem.* **269**, 849–859.

Han, J- S., Kim, H- C., Chung, J- K., Kang, H- S., Donaldson, Z., and Koh, J- K. (1998) The potential role for CDC42 protein from rat brain cytosol in phospholipase D activation. *Biochem. Mol. Biol. Int.* **45**, 1089–1103.

Inoue, H., Shimooku, K., Akisue, T., and Nakamura, S. (1995) Potential role of protein phosphorylation in GTP-gamma-S-dependent activation of phospholipase D. *Biochem. Biophys. Res. Commun.* **210**, 542–548.

James, G. and Olson, E. N. (1990) Fatty acylated proteins as components of intracellular signaling pathways. *Biochemistry* **29**, 2623–2634.

Kawamura, S. and Murakami, M. (1991) Calcium-dependent regulation of cyclic GMP phosphodiesterase by a protein from frog retinal rods. *Nature* **349**, 420–423.

Kawamura, S., Takamatsu, K., Saitoh, S., and Noguchi, T. (1992) Purification and characterization of S-modulin, a calcium-dependent regulator on cGMP phosphodiesterase in frog rod photoreceptors. *Biochem. Biophys. Res. Commun.* **186**, 411–417.

Kiss, Z. and Anderson, W. H. (1994) Selective down-regulation of protein kinase c-epsilon by carcinogens does not prevent stimulation of phospholipase D by phorbol ester and platelet-derived growth factor. *Biochem. J.* **300**, 751–756.

Kobayashi, M., Takamatsu, K., Saitoh, S., Miura, M., and Noguchi, T. (1992) Molecular cloning of hippocalcin, a novel calcium-binding protein of the recovering family exclusively expressed in hippocampus. *Biochem. Biophys. Res. Commun.* **189**, 511–7.

Kobayashi, M., Takamatsu, K., Saitoh, S., and Noguchi, T. (1993) Myristoylation of hippocalcin is linked to its calcium-dependent membrane association properties. *J. Biol. Chem.* **268**, 18898–18904.

Kuno, T., Kajimoto, Y., Hashimoto, T., Mukai, H., Shirai, Y., Sajeki, S., and Tanaka, T. (1992) cDNA cloning of a neural visinin-like Ca(2+)-binding protein. *Biochem. Biophys. Res. Commun.* **184**, 1219–1225.

Kutuzov, M. A., Shmukler, B. E., Suslov, O. N., Dergachev, A. E., Zargarov, A. A., and Abdulaev, N. G. (1991) P26-calcium binding protein from bovine retinal photoreceptor cells. *FEBS Lett.* **293**, 21–24.

Liscovitch, M., Chalifa, V., Pertile, P., Chen, C. S., and Cantley, L. C. (1994) Novel function of phosphatidylinositol 4,5-bisphosphate as a cofactor for brain membrane phospholipase D. *J. Biol. Chem.* **269**, 21403–21406.

Malcolm, K. C., Ross, A. H., Qiu, R. G., Symons, M., and Exton, J. H. (1994) Activation of rat liver phospholipase D by the small GTP-binding protein RhoA. *J. Biol. Chem.* **269**, 25951–25954.

Massenburg, D., Han, J. S., Liyanage, M., Patton, W. A., Rhee, S. G., Moss J., and Vaughan, M. (1994) Activation of rat brain phospholipase D by ADP-ribosylation factors 1,5, and 6: separation of ADP-ribosylation factor-dependent and oleate-dependent enzymes. *Proc. Natl. Acad. Sci. USA* **91**, 11718–11722.

McGinnis, J. F., Stepanik, P. L., Baehr, W., Subbaraya, I., and Lerious, V. (1992) Cloning and sequencing of the 23 kDa mouse photoreceptor cell-specific protein. *FEBS Lett.* **302**, 172–176.

Mohn, H., Chalifa, V., and Liscovitch, M. (1992) Substrate specificity of neutral phospholipase D from rat brain studied by selective labeling of endogenous synaptic membrane phospholipids *in vitro*. *J. Biol. Chem.* **267**, 11131–11136.

Pertile, P., Liscovitch, M., Chalifa, V., and Cantley, L. C. (1995) Phosphatidylinositol 4,5-bisphosphate synthesis is required for activation of phospholipase D in U937 cells. *J. Biol. Chem.* **270**, 5130–5135.

Polans, A. S., Buczylko, J., Crabb, J., and Palczewski, K. (1991) A photoreceptor calcium binding protein is recognized by autoantibodies obtained from patients with cancer-associated retinopathy. *J. Cell Biol.* **112**, 981–989.

Randazzo, P. A., Terui, T., Sturch, S., Fales, H. M., Ferrige, A. G., and Kahn, R. A. (1995) The myristoylated amino

terminus of ADP-ribosylation factor 1 is a phospholipid- and GTP-sensitive switch. *J. Biol. Chem.* **270**, 14809–14815.

Shin, I., Kweon, S-M., Lee, Z-W., Kim, S. I., Joe, CO., Kim, J-H., Park, Y-M., and Ha, K-S. (1999) Lysophosphatidic acid increases intracellular H_2O_2 by phospholipase D and RhoA in rat-2 fibroblasts. *Mol. Cells* **9**, 292–299.

Siddiqi, A. R., Smith, J. L., Ross, A. H., Qiu, R. G., Symons, M., and Exton, J. H. (1995) Regulation of phospholipase D in HL60 cells. Evidence for a cytosolic phospholipase D. *J. Biol. Chem.* **270**, 8466–8473.

Singer, W. D., Brown, H. A., Bokoch, G. M., and Sternweis, P. C. (1995) Resolved phospholipase D activity is modulated by cytosolic factors other than Arf. *J. Biol. Chem.* **270**, 14944–14950.

Sommer, D. and Song, P. S. (1994) Isolation and purification of a small-molecular-weight GTP-binding protein from plants. *Protein Express Purif* **5**, 402–408.

Takamatsu, K., Kitamura, K., and Noguchi, T. (1992) Isolation and characterization of recoverin-like Ca(2+)-binding protein from rat brain. *Biochem. Biophys. Res. Commun.* **183**, 245–251.

Terasawa, M., Nakano, A., Kobayashi, R., and Hidaka, H. (1992) Neurocalcin: a novel calcium-binding protein from bovine brain. *J. Biol. Chem.* **267**, 19596–19599.

Towler, D. A., Gordon, J. I., Adams, S. P., and Glaser, L. (1988) The biology and enzymology of eukaryotic protein acylation. *Annu. Rev. Biochem.* **57**, 69–99.

Uings, I. J., Thompson, N. T., Randall, R. W., Spacey, G. D., Bonser, R. W., Hudson, A. T., and Garland, L. G. (1992) Tyrosine phosphorylation is involved in receptor coupling to phospholipase D but not phospholipase C in the human neutrophil. *Biochem. J.* **281**, 597–600.

Zozulya, S. and Stryer, L. (1992) Calcium-myristoyl protein switch. *Proc. Natl. Acad. Sci. USA* **89**, 11569–11573.