Collapsin Response Mediator Protein-2 Inhibits Neuronal Phospholipase D2 Activity by Direct Interaction*

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Running title; CRMP-2-induced PLD2 inhibition via direct interaction
SUMMARY

Although the functional significance of neuronal phospholipase D (PLD) is being recognized, little is known about its regulatory role in neuronal cells. To elucidate the regulatory mechanism of neuronal PLD, we investigated PLD2-binding neuronal protein from rat brain cytosol. During the fractionation of rat brain cytosol by four column chromatography, a 62 kDa PLD2-interacting protein was detected by PLD2-overlay assay and identified as collapsin response mediator protein-2 (CRMP-2), which controls the guidance and outgrowth of neuronal axon. Using bacterially expressed glutathione S-transferase fusion proteins, we found that two regions (amino acids 65-192, the phox domain, and 724-825) of PLD2 and a single region (amino acids 243-300) of CRMP-2 are required for the direct binding of both proteins. A co-immunoprecipitation study in COS-7 cells also showed an in vivo interaction between CRMP-2 and PLD2. Interestingly, CRMP-2 was found to potently inhibit PLD2 activity in a concentration-dependent manner (IC50=30 nM). Overexpression studies also showed that CRMP-2 is an in vivo inhibitor of PLD2 in PC12 cells. Moreover, increasing the concentration of Semaphorin 3A, one of the repulsive axonal guidance cues, showed that PLD2 activity can be inhibited in PC12 cells. Immunocytochemistry further revealed that PLD2 is co-localized with CRMP-2 in the distal tips of neurites, its possible action site, in differentiated PC12 cells. Taken together, our results indicate that CRMP-2 may interact directly with and inhibit neuronal PLD2, suggesting that this inhibitory mode of regulation may play a role in neuronal pathfinding during the developmental stage.
INTRODUCTION

Phospholipase D (PLD)\(^1\) catalyzes the hydrolysis of phosphatidylcholine (PC) to generate phosphatidic acid (PA) and choline in response to various signals, including hormones, neurotransmitters, and growth factors (1). PA has been shown to be involved in multiple physiological events in various cells, including mitogenesis, vesicle trafficking, respiratory burst in immune cells, and actin cytoskeletal rearrangements (2-5). These relationships mean that receptor-mediated PLD activity is deeply implicated in a variety of cellular responses in different cells.

A number of reports have suggested that neuronal PLD activity may be closely associated with amyloid β-induced cytotoxicity, hormone secretion, and neuronal differentiation (6-9). Recently, the two types of mammalian PLD isozymes, PLD1 and PLD2, were cloned. These isozymes have a sequence homology exceeding 50%, and contain several domains (conserved regions I, II, III, and IV) that are highly conserved in the PLD superfamily, and are dependent on PIP\(_2\) for their activity. PLD1 can be activated by many cytosolic factors, including protein kinase C (PKC) and small GTP-binding proteins such as Rac1, Cdc42, ARF1, and RhoA (10-15). On the other hand, PLD2 activity in the same setting is relatively insensitive to the PLD1-activating factors (16-17). However, the manner in which agonist-induced PLD isozymes are regulated in neuronal cells remains substantially unknown, though it has been reported that PLD mRNA and protein are more highly expressed in brain than other tissue (18-20). In this regard, we believed an investigation of neuronal PLD regulation was warranted to further determine its function.

Collapsin response mediator protein-2 (CRMP-2) is a cytosolic phosphoprotein that is exclusively expressed in the nervous system (21). It has been suggested to be a key mediator of Semaphorin 3A action, one of the repulsive guidance cues, that leads to growth cone collapse during neuronal axon guidance (22). In contrast to the rapid progress made in identifying and
characterizing axon guidance molecules and their receptors (34-36, 55-57), much remains to be discovered about the intracellular mechanism by which signals are transduced into the eventual response of the growth cone.

In our present study, we found for the first time that collapsin response mediator protein-2 (CRMP-2) specifically inhibits PLD2 activity by direct interaction. In addition, we tried to elucidate the role of CRMP-2 on the regulation of PLD2 in neuronal cells using PLD2-overexpressing PC12 cells as a model system. Thus, it is hoped that this study will provide several clues concerning the involvement of PLD2 in growth cone collapse.

**EXPERIMENTAL PROCEDURES**

**Materials** - V8 protease, dipalmitoylphosphatidylinositol [methyl-³H] choline, chelating-Sepharose, Q-Sepharose, phenyl-Sepharose, HiTrap Heparin column, and the enhanced chemiluminescence kit (ECL system) were purchased from Amersham Pharmacia Biotech. Horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgA, IgM, and IgG were from Kirkegaard & Perry Laboratories (Gaithersburg, MD). The Bio-Gel HT column and the polyvinylidene difluoride membrane were from Bio-Rad. Anti-actin antibody was from ICN pharmaceuticals (Costa Mesa, USA). β-octylglucopyranoside was obtained from Calbiochem. Dipalmitoylphosphatidylcholine, phosphatidylinositol-4, 5-bisphosphate, dioleoylphosphatidylethanolamine, paraformaldehyde, poly-L-lysine, anti-flag M2 agarose, anti-flag antibody, TRITC-conjugated goat anti-mouse IgG, and FITC-conjugated goat anti-rabbit IgG were from Sigma. Dulbecco’s modified Eagle’s medium was from Life Technologies, Inc. Anti-neuropilin-1 polyclonal antibody was from Oncogene Research Products (Cambridge, MA). Immobilized protein A was from Pierce. A polyclonal antibody that recognizes both PLD1 and PLD2 was generated as described previously (23).
**Preparation of rat brain cytosol** - All preparations were performed at 4°C in a refrigerated room or on ice. Adult rat brains (total of 30 g) were homogenized using a polytron homogenizer in a homogenation buffer containing 20 mM Tris, pH 7.6, 1 mM MgCl₂, 1 mM PMSF, and 0.1 mM DTT. The homogenate was centrifuged at 100,000 x g for 1 hr and the resulting supernatant (cytosolic fraction) was collected.

**Purification of the PLD2-interacting protein** - The cytosolic fraction (900 mg) was applied to a Q-Sepharose anion exchange column (13 cm X 3 cm) equilibrated with buffer A (20 mM Tris, pH 7.6, 1 mM EDTA, 1 mM EGTA and 0.1 mM DTT). Unbound proteins (the flow through fractions) were collected and NaCl was added as salt to 2 M. After centrifugation (50,000 x g, 20 min), the resulting supernatant was loaded onto a phenyl-Sepharose column (70 cm x 2 cm). Proteins were eluted at a flow rate of 2 ml/min by applying a decreasing gradient of NaCl (2 to 0 M) over 60 min. Fractions were collected and tested by PLD2 overlay assay. Peak fractions were pooled, and diluted with buffer A to adjust the salt concentration to 50 mM NaCl, and then loaded onto a HiTrap heparin column (1 ml, Pharmacia) equilibrated with buffer A containing 50 mM NaCl. Bound proteins were eluted at a flow rate of 0.5 ml/min using a linear gradient of 0.05-1 M NaCl over 30 min. Fractions were collected and also tested by PLD2 overlay assay. Fractions containing PLD2-interacting proteins were pooled and continuously loaded onto a Bio Gel HT (1 ml, Bio-Rad) equilibrated with buffer B (20 mM Tris, pH 7.6, 50 mM NaCl, 0.1 mM DTT). Bound proteins were then eluted at a flow rate of 0.3 ml/min using an increasing gradient of KH₂PO₄ (0-0.25 M). Fractions were then collected and tested by PLD2 overlay assay. The fractions containing p62 were collected and stored at -70°C for further study.

**Purification of recombinant PLD2 from baculovirus-transfected sf9 cell** - Hexa-histidine (His₆)-tagged human PLD2 was purified from detergent extracts of baculovirus-infected sf9 cells by Chelating Sepharose affinity column chromatography as described previously (15).
**PLD2 overlay assay** - The assay method used was a modification of one described previously (24). In brief, rat brain cytosolic proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The blots were preincubated overnight with PLD assay buffer (50 mM HEPES, pH 7.3, 3 mM EGTA, 3 mM CaCl₂, 3 mM MgCl₂, and 80 mM KCl) containing 0.1 mM DTT and 5% (w/v) skimmed milk at room temperature, and re-incubated with the same buffer containing 1 µg/ml of purified PLD2 for 3 hr at room temperature. The membranes were then washed several times with Tris-buffered saline (20 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 0.05% Tween 20 and reacted with polyclonal antibodies directed against PLD for 3 hr. After washing with TTBS, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies for 1 hr and developed using an enhanced chemiluminescence kit as described by the manufacturer.

**Identification of 62 kDa protein by peptide sequence analysis** - Purified PLD2-interacting protein from the hydroxyapatite column was digested for 2 hr at 37°C with V8 protease obtained from *Staphylococcus aureus* and then run on 15% SDS-PAGE to separate the cleaved peptides. After transferring the peptides to a polyvinylidene difluoride membrane, they were stained with Coomassie brilliant blue, rinsed several times with 30% methanol, excised, and subjected to Edman degradation. The candidate protein was identified by sequencing (ABI473 Sequencer) at the Institute of Basic Science in Busan, Korea, and by comparing the results obtained with the Swiss-Prot protein database using the BlastP algorithm.

**In vitro binding analysis** - Affinity-purified PLD2 antibodies immobilized with protein A beads were first incubated with purified PLD2 for 2 hr at 4°C. After a brief centrifugation, the precipitates were re-incubated with the indicated amounts of purified CRMP-2 for 15 min at 37°C in PLD assay buffer containing 1% TX-100. Binding site mapping between PLD2 and CRMP-2 was performed by incubating the indicated amounts of GST fusion proteins with purified PLD2 or
rat CRMP-2 respectively in the same buffer condition for 15 min at 37°C. After a brief centrifugation, the pellets were washed three times with the same buffer before being loaded onto a polyacrylamide gel.

**Vector construction and preparation of glutathione-S-transferase fusion protein** - The full-length coding region of human CRMP-2 was ligated into the EcoRI site of pGEX4T1 vector to make a GST fusion protein (Amersham Pharmacia Biotech). To construct the deletion mutants of CRMP-2, the full-length coding region of human CRMP-2 was also digested into N- and C-terminal serial deletion mutants by random cleavage. The deletion mutants were then ligated into the 5’ Eco RI and the 3’ SalI site of the same vector. The phox (PX) and pleckstrin homology (PH) domains were constructed by digesting the PCR products using the restriction enzymes, 5’ EcoRI and 3’ SalI, and inserted into pGEX-4T1 vector. pCMV vector was used as the mammalian expression vector of CRMP-2. The full-length coding region of human CRMP-2 was ligated into the 5’ Eco RI and the 3’ XbaI sites of pCMV vector (Clontech). Standard methods were used for subcloning and polymerase chain reaction (PCR) to produce expression vectors encoding the respective GST fusion proteins (25). *Escherichia coli* BL21 cells were transfected with individual expression vectors encoding the GST fusion proteins and grown. The GST fusion proteins so obtained were purified by standard methods (26) using glutathione-Sepharose 4B (Amersham Pharmacia Biotech).

**In vitro PLD activity assay** - The PLD activity assay was performed by measuring choline release from phosphatidylcholine, as described previously (27) but with minor modification. In brief, the reaction was carried out at 37°C for 15 min in a 125 µl assay mixture containing PLD assay buffer, the PLD preparation, and 25 µl of phospholipid vesicles composed of dioleoylphosphatidylethanolamine, PIP2, and dipalmitoylphosphatidylcholine in a molar ratio of 16:1.4:1 and dipalmitoylphosphatidyl-[methyl-3H]choline (a total 150,000 cpm/assay). The reactions were
terminated by adding 0.3 ml of a solution containing 1 N HCl and 5 mM EGTA and 1 ml of chloroform: methanol: HCl (50: 50: 0.3). After a brief centrifugation, the amount of [methyl-\(^{3}\)H]choline in 0.5 ml of the aqueous phase was quantified by liquid scintillation counting.

**Cell culture** - PLD2-overexpressing PC12 cells were prepared using the tetracyclin-regulated (TET - OFF) expression system (Life Technologies, Inc.) as described previously (28). Clonal cell lines were usually maintained in Dulbecco’s modified Eagle’s medium supplemented with 0.5 µg/ml tetracycline, 10% (v/v) equine serum, and 5% fetal bovine serum. The cells were differentiated by incubating them in Dulbecco’s modified Eagle’s medium containing 0.5% fetal bovine serum and nerve growth factor (100 ng/ml) for 4 days. The COS-7 cells were maintained in a growth medium composed of Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37°C in a humidified, CO₂-controlled (5%) incubator. To induce the transient expression of human PLD2 or human CRMP-2, COS-7 or PC12 cells were plated at densities of 1 x 10⁶ cells/well in 100-mm dishes and at 1 x 10⁵ cells/well in 6 well plates respectively and transfected using LipofectAMINE (Life Technologies, Inc.) as described previously (29).

**Co-immunoprecipitation** - After transfecting COS-7 cells with the cDNAs of human PLD2 or human CRMP-2, the cells were harvested and lysed with brief sonication in PLD assay buffer containing 1% TX-100, 1 mM PMSF, 1 µg/ml leupeptin, and 5 µg/ml aprotinin. The cells were then centrifuged at 100,000 x g for 1 hr and the resulting supernatant was incubated with anti-flag M2 agarose for 4 hr at 4°C. After three washings with the same incubation buffer, the final pellet was loaded onto a polyacrylamide gel for immunoblot analysis.

**Immunoblot analysis** - Proteins were denatured by boiling for 5 min at 95°C in Laemmli sample buffer (30), separated by SDS-PAGE, and transferred to nitrocellulose membranes by electroblotting using the Bio-Rad wet transfer system (Hercules, USA). After blocking in TTBS buffer (10 mM Tris/HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) containing 5% skim milk
powder, the membranes were incubated with individual monoclonal or polyclonal antibodies, and then further incubated with anti-mouse or anti-rabbit IgG coupled to horseradish peroxidase. Blots were detected using an enhanced chemiluminescence kit according to the manufacturer’s instructions.

**Immunocytochemistry** - Immunocytochemistry was performed based on a procedure reported previously (31). In brief, flag-CRMP-2 transfected PLD2-overexpressing PC12 cells in the presence of nerve growth factor were grown on coverslips for 4 days, rinsed with phosphate-buffered saline (PBS) four times, and fixed with 4% (w/v) paraformaldehyde overnight at 4°C. After rinsing with PBS and blocking with PBS containing 1% goat serum and 0.1% TX-100 for 30 min at room temperature, the cells were incubated with 2 µg/ml of primary polyclonal and monoclonal antibody specific to PLD and flag for 2 hr at room temperature. After washing six times with PBS, FITC-labeled goat anti-rabbit antibody and TRITC-conjugated goat anti-mouse antibody were incubated with the cells for 1 hr to allow the visualization of PLD2 and CRMP-2. After six washings with PBS, the slides were examined under confocal microscope (Zeiss, Denmark).

**In vivo PLD activity** - In vivo PLD activity was determined as described earlier (32). In brief, PLD2-overexpressing PC12 cells were cultured in the presence or absence of tetracycline for 48 hr. The cells were loaded with [3H]myristic acid (2 µCi/ml) for 4 hr and then washed twice with Dulbecco’s modified Eagle’s medium. The loaded cells were then incubated with 0.4% butanol for 5 min in the presence of the indicated concentrations of Semaphrin 3A, scraped into 0.8 ml of methanol and 1M NaCl (1:1), and mixed with 0.4 ml of chloroform. The organic phases were dried and the lipids were separated by thin layer chromatography on silica gel plates. The PLD activity of the vector or human CRMP-2 transfected PLD2-overexpressing PC12 cells was determined using the same procedure. The amount of [3H] phosphatidylbutanol (PBtOH) formed was
expressed as percentage of the total [³H]-lipid to account for cell labeling efficiency differences.

RESULTS

A 62 kDa PLD2-interacting protein was purified from rat brain cytosol - Cytosolic fraction (900 mg of proteins) of rat brain was fractionated by sequential column chromatography and the fractions so obtained were subjected to a blot overlay assay using PLD2 to explore the major PLD2-binding proteins. Initially, the flow-through fraction from the Q-Sepharose column was subjected to phenyl-Sepharose column chromatography. A blot overlay assay showed that the proteins with relative molecular masses of 62 kDa (p62) and 40 kDa (p40), which co-eluted in a linear gradient of 1.8-1.6 M NaCl from the phenyl-Sepharose column, were major PLD2-binding proteins (data not shown). A further purification step was performed to purify the PLD2-binding proteins on a Hitrap Heparin column. p62 and p40 were also co-eluted in 0.2 M NaCl as determined by overlay assay but other minor bands were removed by column chromatography (data not shown). Using a hydroxyapatite column, p62 and p40 were separately eluted by 0.15-0.2 M and 0.2-0.25 M KH₂PO₄, respectively (Fig. 1A). SDS-PAGE showed that the p62 yield was 0.7 mg, assuming proportionality between the band densities and the protein quantities after Coomassie Brilliant Blue staining (Fig. 1B). The p62 protein was also detected in the overlay blot (Fig. 1C). These results suggest that the 62 kDa protein purified from rat brain cytosol binds directly with PLD2.

The 62 kDa protein was identified as rat CRMP-2 - The identity of p62 in fractions containing nearly homogenous 62 kDa protein (>90%) was determined in two ways. First, the p62 peptide produced by V8 protease was sequenced and its N-terminal sequence identified as
KKNIPRITSDDLLIK. This sequence was then searched for in the Swiss-Prot protein database using the BlastP peptide program. The search showed that this sequence was almost identical with an internal sequence (amino acids 6-20) of the rat collapsin response mediator protein (rCRMP), especially of CRMP-2 and -4 (Fig. 2A). However, CRMP-4 has an apparent molecular mass of 39 kDa, which didn’t match the purified p62, and which suggested that p62 might be a CRMP-2. p62 was then identified as CRMP-2 by immunoblot analysis using monoclonal antibody specific to CRMP-2 (Fig. 2B). Therefore, we suggest that the 62 kDa protein identified as a PLD2-direct binder is a rCRMP-2.

**CRMP-2 interacts directly with two regions (amino acids 65-192 and 724-825) of PLD2** - *In vitro* binding and immunoblot analysis were used to determine whether CRMP-2 associates directly with PLD2. As shown in Fig. 3A, purified rCRMP-2 interacted specifically with PLD2, but not with the anti-PLD antibody-protein A Sepharose complexes alone, confirming a direct interaction between CRMP-2 and PLD2. To identify the region in PLD2 responsible for this CRMP-2 binding, we constructed and prepared GST fusion proteins of PLD2 fragments, as indicated in Fig. 3B. The GST fusion proteins were then tested for their ability to bind to rCRMP-2. As shown in Fig. 3C, rCRMP-2 interacted with both the N-terminal (amino acids 1-314) and the C-terminal (amino acids 724-825) regions of PLD2, whereas no interaction was observed with the other regions. To identify the PLD2 N-terminal site responsible for CRMP-2 binding, we constructed GST-tagged phox (PX) and pleckstrin homology (PH)-like domain for *in vitro* binding analysis. The results obtained showed that CRMP-2 binds to the PX domain (amino acids 65-192) in the N-terminal region of PLD2 (Fig. 3D). These results suggest that two regions (amino acids 65-192 and 724-825) of PLD2 are responsible for its binding to CRMP-2.

**Amino acids 243-300 of CRMP-2 may be essential for its direct interaction with PLD2** - To identify the region of CRMP-2 responsible for binding to PLD2, we generated N- and C-terminal
deletion mutants of human CRMP-2 (hCRMP-2). It should also be noted that hCRMP-2 is almost identical to rCRMP-2 (99% sequence identity) (33). As shown in Fig. 4A, N- and C-terminal deletion mutants were constructed by the stepwise removal of hCRMP-2. Wild-type hCRMP-2 and all of its deletion mutants were tagged to GST, affinity-purified and examined in terms of their ability to bind to PLD2. The results obtained using C-terminal deletion mutants showed that PLD2 interacted directly with C2(1-300) of hCRMP-2 but that not with C3(1-143) of hCRMP-2. This suggests that the amino acids 144-300 of hCRMP-2 may be important for binding to PLD2 (Fig. 4B). The purity and the quantity of the C-terminal deletion mutants of hCRMP-2 were verified by Ponceau staining (lower panel of Fig. 4B). To further identify the PLD2 binding site in CRMP-2, GST-tagged N-terminal serial deletion mutants of hCRMP-2 were subjected to a pull-down assay and immunoblot analysis. Fig. 4C shows that the amino acids 243-300 region of hCRMP-2 is involved in the binding of PLD2. Our results suggest that a region (amino acids 243-300) of CRMP-2 may be important for PLD2 binding.

**PLD2 interacts specifically with CRMP-2 in COS-7 cells** - To verify that PLD2 and CRMP-2 interact in vivo, we co-transfected the cDNAs of hCRMP-2 and hPLD2 into COS-7 cells and performed immunoblot analysis after immunoprecipitating flag-tagged hCRMP-2 using anti-flag antibodies. As shown in Fig. 5, PLD2 was found to interact specifically with CRMP-2 in these cells, suggesting that PLD2 forms a complex with CRMP-2 in COS-7 cells.

**CRMP-2 inhibits PLD2 activity in a concentration-dependent manner** - We next examined whether CRMP-2 plays a role in the regulation of PLD2, by measuring the effect of CRMP-2 on PLD2 activity in vitro. As shown in Fig. 6, purified rCRMP-2 was found to specifically inhibit PLD2 activity in a concentration-dependent manner. Under these conditions, the IC₅₀ of this CRMP-2-mediated inhibition was about 30 nM, and inhibition was complete at 150 nM. To further evaluate the effect of CRMP-2 on PLD2 activity, the GST-tagged wild-type and the deletion
mutants, N3(243-573) and N4(301-573), of hCRMP-2 were reconstituted in a PLD activity assay. As expected, N3(243-573) showed an inhibitory potency comparable to that of wild-type hCRMP-2. However, GST alone or N4(301-573) did not inhibit PLD2 activity, although they were added to the assay up to 150 nM, at which level PLD2 activity was completely inhibited by wild-type hCRMP-2 (Fig. 6). This result is consistent with the in vitro binding analysis (Fig. 4B and C). In summary, we suggest that amino acids 243-300 of CRMP-2 may be essential for binding to and inhibiting PLD2.

**Overexpression of CRMP-2 inhibits PLD2 activity in PC12 cells** - To determine the effect of CRMP-2 in neuronal cells, we transfected hCRMP-2 in PLD2-overexpressing PC12 cells, which were able to induce PLD2 expression upon tetracycline withdrawal (28), and measured PLD activity in vivo. As is shown in Fig. 7A, PLD2 activity was inhibited by the transfection of hCRMP-2 cDNA, but not by that of the vector only. To determine the transfection efficiency of single gene in this cell line, the cDNA of green fluorescent protein was transfected and its expression confirmed by conventional epifluorescence analysis. As a result, these cells were found to have a transfection efficiency of approximately 40% (data not shown). At this stage, we also checked whether transfecting with CRMP-2 affects the expression of PLD2 in PC12 cells. As is shown by Fig. 7B, the overexpression of CRMP-2 was found to have no effect on the expression of PLD2 in PC12 cells. Therefore, these results suggest that CRMP-2 may be an in vivo inhibitor of PLD2 in PC12 cells.

**Semaphorin 3A inhibits PLD2 activity in PC12 cells in a concentration-dependent manner** - It has been suggested that CRMP-2 may be an intracellular mediator of Semaphorin 3A, a chemorepulsive axonal guidance molecule, and can be involved in inducing the collapse of the growth cone in neuronal cells (22). To verify the effect of Semaphorin 3A on the in vivo activity of PLD2, we first confirmed the expression of neuropilin-1, a member of the Semaphorin 3A receptor
complex (34-36), in PLD2-overexpressing PC12 cells by immunoblot analysis. The result obtained showed that a significant amount of neuropilin-1 was endogenously expressed in the cells and that this was unaffected by either the absence or presence of tetracycline (inset of Fig. 8). On treating PC12 cells with Semaphorin 3A, PLD2 activity was inhibited in a concentration-dependent manner (Fig. 8), suggesting that CRMP-2 may be involved in Semaphorin 3A-induced PLD2 inhibition.

**PLD2 is co-localized with CRMP-2 in the distal tips of neurites in differentiated PC12 cells** - To demonstrate the localization of PLD2 in differentiated PC12 cells, PLD2-inducible PC12 cells were cultured in nerve growth factor-containing media in the absence or in the presence of tetracycline and examined immunocytochemically using anti-PLD antibody. In PLD2-induced cells, the pattern of PLD2 immunofluorescence was distributed in membrane regions, which included the plasma membrane, the neurite membrane and the distal tips of neurites. However, no PLD2 immunofluorescence was detected in cells cultured in the presence of tetracycline, which showed that PLD2 expression is the result of PLD2 induction (Fig. 9A). To further confirm the co-localization of PLD2 and CRMP-2 in differentiated PC12 cells, we transfected flag-tagged hCRMP-2 in PC12 cells, which was then differentiated with nerve growth factor, and immunostained with anti-PLD and anti-flag antibody. As expected, CRMP-2 was found to be co-localized with PLD2 in the distal tips of neurites in differentiated PC12 cells (Fig. 9B). This result suggests that PLD2 is localized in the distal tips of neurites in differentiated PC12 cells and that, in this location, it may be negatively regulated by CRMP-2.
DISCUSSION

Although some evidence is available about the expression and signaling pathway of PLD, the molecular regulatory mechanism that modulates its role in neuronal cells has not been elucidated. Thus, we investigated PLD2-binding neuronal protein from rat brain using a PLD2-overlay assay and demonstrated that collapsin response mediator protein-2 (CRMP-2) interacts directly with PLD2 to inhibit its activity. In the present study, we also investigated the use of PLD2-overexpressing PC12 cells as a model system, in an effort to outline the molecular regulation and function of neuronal PLD2. Furthermore, by using Semaphorin 3A, one of the repulsive guidance cues, the possible relevancies of PLD2 and CRMP-2 in neuronal cells were identified.

In the present paper, we report for the first time that CRMP-2 interacts directly with PLD2 and inhibits its activity. Our several lines of investigation show that CRMP-2 acts as a potent inhibitor of PLD2 in cells as follows. First, CRMP-2 purified from rat brain was found to inhibit PLD2 activity in a concentration-dependent manner \textit{in vitro} (IC$_{50}$ = 30 nM) (Fig. 6). Second, GST-CRMP-2 was also found to inhibit PLD2 activity with a potency similar to that of CRMP-2 purified from rat brain (Fig. 6). Third, the deletion mutant N3(243-573) of CRMP-2 was found to inhibit PLD2 activity to a similar extent as the wild-type, whereas the further deletion mutant N4(301-573) did not inhibit PLD2 activity (Fig. 6), suggesting that the 243-300 amino acid region of CRMP-2 may be responsible for PLD2 inhibition. Fourth, the transfection of CRMP-2 in COS-7 was found to inhibit PLD2 activity (data not shown). Fifth, the overexpression of CRMP-2 in PC12 cells also inhibits PLD2 activity (Fig. 7). These results suggest that CRMP-2 plays an inhibitory role in the regulation of PLD2 in neuronal cells.

CRMP-2 interacts directly with both the N-terminal (amino acids 65-192; the phox domain) and the C-terminal (amino acids 724-825) regions of PLD2 (Fig. 3B). This is supported by reports,
which found that the integrity of the N-and C-terminal regions is necessary for the enzymatic activity of PLD and that the two copies of the HKD domains may be brought together to form an active catalytic site by self-association (37-38). In addition, CRMP-2 has WD repeat-like domain in the C-terminus (39), which is not strictly identical to but similar to the WD repeat. Moreover, the WD repeat is involved in various protein-protein interactions, such as the formation of the α and βγ complexes of trimeric G protein (40-41). Wang et al reported that brain CRMPs might form oligomers in vivo and that the WD repeat domain is likely to participate in the CRMP-CRMP interaction. Intriguingly, this domain in CRMP-2 does not overlap the PLD2 interacting site (amino acids 243-300). This evidence leads us to hypothesize that oligomeric CRMP-2 interacts directly with the N- and/or the C-terminals of self-associated PLD2, and that by binding to this region CRMP-2 interferes with the formation of the PLD2 catalytic center, and thereby inhibits PLD2. However, much of the detailed mechanism of the regulation of PLD2 by CRMP-2 remains to be elucidated.

CRMP-2 is a cytosolic phosphoprotein but some portion of the protein is known to be associated with brain membranes (21). In our previous report, we found that PLD2 is localized near the plasma membrane of PC12 cells (26). The fact that both proteins exist in the neuronal membrane implies that in this location, they may associate and play a key role in neuronal functions. In the present study, it proved technically difficult to detect the Semaphorin 3A-dependent association of CRMP-2 and PLD2 in PC12 cells using a co-immunoprecipitation approach, because the expression of CRMP-2 in PC12 cells is relatively low compared to that in hCRMP-2-transfected COS-7 cells. However, we demonstrated for the first time using co-immunoprecipitation that CRMP-2 interacts specifically with PLD2 in COS-7 cells, thus demonstrating the in vivo interaction between CRMP-2 and PLD2 (Fig. 5). Furthermore, this demonstration of the in vitro association of both proteins also provides strong support for the
interaction of CRMP-2 and PLD2 in cells. To date, little has been elucidated as to how CRMP-2 is involved in the Semaphorin 3A-dependent signaling pathway. However, our findings present the possibility that CRMP-2 may form a complex with PLD2 in cells and that this may be involved in Semaphorin 3A-related neuronal functions.

CRMP-2 is expressed exclusively in the nervous system (21-22). Accumulated evidence shows that CRMP-2 is a critical molecule, which induces the growth cone collapse of neuronal cells. First, CRMP-2 bears 37% sequence homology with Unc-33 (33), a nematode protein, the absence of which produces aberrant elongation of axons and uncoordinated movement in the worm Caenorhabditis elegans (42). Second, CRMP-2 is almost identical to chick CRMP-62 (98% sequence homology) (33), first identified by its possible involvement in the Semaphorin 3A/collapsin-1-induced mediation of growth cone collapse in chick dorsal root ganglion neurons (22). More recently, it was reported that CRMP-2 is deeply involved in lysophosphatidic acid (LPA)-induced growth cone collapse of the dorsal root ganglion neurons (43). These findings suggest that CRMP-2 may act as an intracellular key molecule in the collapse of growth cones in neuronal cells during the developmental stage.

Neuronal migration during the developmental stage is regulated by extracellular axon guidance molecules, and is also dependent on the dynamics of the actin cytoskeleton, normally present in the filopodia and lamelipodia of growth cones (44-45). In this study, we suggest that PLD2 activity may be involved in the regulation of the dynamics of the actin cytoskeleton in the growth cone membrane of neurons. Our immunocytochemical findings show that some portion of PLD2 in differentiated PC12 cells is located in the distal tips of neurites, which are recognized as major CRMP-2 action sites (22, 46), and that in that location, PLD2 is co-localized with CRMP-2 (Fig. 9B). These findings further support the likelihood of its role in this location. Moreover, many reports have suggested a close association between PLD and the actin cytoskeleton (24, 47-51). In
a previous report, we demonstrated that β-actin interacts directly with PLD2 in vitro and that the actin cytoskeleton is co-localized with PLD2 in PC12 cells (26) and in differentiated PC12 cells, we also observed co-localization of F-actin and PLD2 in the distal tips of neurites using immunocytochemical approach (data not shown). Moreover, lysophosphatidic acid-stimulated PLD activity plays a role in actin polymerization including the formation of stress fiber in various cell types, such as fibroblasts and endothelial cells (5, 52-54). Therefore, the evidence accumulated to date suggests that the PLD2 located in the growth cone membrane may be closely associated with the actin cytoskeleton and that its activity may be important for actin cytoskeletal rearrangement.

Semaphorin 3A is a chemorepulsive axonal guidance molecule that binds to its specific receptor, a plexin-neuropilin-1 complex, on the surface of growth cones and thereby induces neuronal growth cone collapse (55-57). It has been shown that Semaphorin 3A-dependent growth cone collapse is accompanied by a rapid reorganization of the actin filaments (58-59). Recently, Aizawa et al also reported that Semaphorin 3A-induced growth cone collapse occurs because of LIM kinase-mediated cofilin phosphorylation leading to actin depolymerization (60). In this regard, the results of our present study suggest the involvement of PLD2 in the Semaphorin 3A-signaling pathway. First, CRMP-2 as a main signal transducer of Semaphorin 3A interacts directly with PLD2, inhibiting its activity. Second, Semaphorin 3A inhibits PLD2 activity in PC12 cells in a concentration-dependent manner (Fig. 8). These observations suggest that Semaphorin 3A-induced PLD2 inhibition may be closely associated with CRMP-2, and that it plays a role in the growth cone collapse resulting from actin depolymerization in the cells.

In summary, we observed for the first time that CRMP-2 interacts directly with PLD2 and inhibits its activity, and that this inhibition was found to be Semaphorin 3A-dependent in PC12 cells. Therefore, we suggest a mode of action for CRMP-2 in neuronal cells during growth cone collapse. Migrating neurons are required to regulate the dynamics of the actin cytoskeleton on
finding an appropriate site and forming the final neural network. Moreover, during this stage, CRMP-2 transduces the Semaphorin 3A signal that regulates PLD activity, and thus, PLD then becomes involved in the regulation of the actin cytoskeletal changes. Although more detailed work is required, we believe that PLD probably has a functional relevance in the growth cone collapse of neuronal cells. We will continue this work by examining in detail the relationships between PLD and growth cone collapse.

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**FOOTNOTES**

1The abbreviations used are as follows: PLD, Phospholipase D; CRMP-2, collapsin response mediator protein-2; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PIP2, phosphatidylinositol-4, 5-bisphosphate; Sf9, Spodoptera frugiperda; EGTA, ethylene glycol bis(β-aminoethyl ether) N, N, N’, N’-tetraacetic acid; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; PC 12 cell, rat pheochromocytoma PC12 cell; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine; NGF, nerve growth factor; PX, phox domain; PMSF, phenylmethylsulfonylfluoride; DTT, dithiothreitol, PVDF, polyvinylidene difluoride.

*Keyword*: Phospholipase D, collapsin response mediator protein-2, inhibition, direct interaction, PC12 cells, semaphorin 3A
FIGURE LEGENDS

**Fig. 1. Purification and detection of PLD2-interacting 62 kDa protein from rat brain.** *A.* Rat brain cytosol (900 mg of protein) was subjected to sequential column chromatography on Q Sepharose, phenyl-Sepharose, HiTrap heparin, and hydroxyapatite columns. Detailed procedures are described under “Experimental Procedures”. In the final hydroxyapatite column chromatography, proteins were fractionated by elution with the indicated linear gradient of KH$_2$PO$_4$. The protein content of the eluted fractions was monitored by measuring absorbance at 280 nm. *B.* The proteins present in aliquots of the hydroxyapatite fractions were visualized by Coomassie Brilliant Blue staining. The positions of the molecular size standards (in kilodaltons) are shown on the left. *C.* The indicated eluted fractions were overlaid without (-PLD2) or with (+PLD2) purified PLD2, as described under “Experimental Procedure”.

**Fig. 2. Identification of p62 as CRMP-2.** *A.* the peptides obtained from p62 as described under “Experimental Procedures” were sequenced. The analyzed sequence of p62 is shown in **bold** and the matching of CRMP sequence is shown *underlined*. *B.* Purified rCRMP-2 was loaded onto a polyacrylamide gel and immunoblot analysis was sequentially performed using monoclonal antibody directed against CRMP-2. Here, the cytosolic fraction (20 µg) of rat brain was used as a positive control.

**Fig. 3. Direct interaction of CRMP-2 with PLD2 in vitro.** *A.* Anti-PLD antibody complexes were first incubated in the absence (-) or presence (+) of purified PLD2 (0.2 µg). After being washed with PLD assay buffer three times, the complexes were re-incubated with purified rCRMP-2 (150 nM) as described under “Experimental Procedures”. The immune complexes were co-immunoprecipitated and subjected to immunoblot analysis using specific antibody against PLD2 and CRMP-2. *B.* PLD2 cDNA was then fragmented into individual domains consisting of F1 (1-
314), F2 (315-475), F3 (476-612), F4 (613-723), F5 (724-825), F6 (826-934), PX (65-192) and PH (201-310). These fragments were cloned as GST fusion proteins, expressed in *E. coli* and purified using glutathione Sepharose beads. The boxes shown represent the regions of highly conserved sequences in PLD. PX, phox; PH, pleckstrin homology; I, II, III, IV, conserved region I, II, III, IV; CT, C-terminal region. C. Equal amounts (2 µg) of GST or of the GST fusion proteins (GST-PLD2 fragments, F1-F6) were incubated with purified CRMP-2 (210 nM), as described under “Experimental Procedures”. The precipitated proteins were subjected to immunoblot analysis using antibodies directed against CRMP-2. GST was used as the control. D. 2 µg of GST or of the GST fusion proteins (F1, PX, PH) were also incubated with the purified CRMP-2 (210 nM), as described under “Experimental Procedure”, and examined by pull-down assay followed by immunoblot analysis. All the results shown are representative of two separate experiments.

**Fig. 4. Identification of the region of CRMP-2 responsible for binding to PLD2.** A. Schematic representation of GST-tagged wild-type CRMP-2 and of its N- and C-terminal deletion mutants (shown as black bars) as used in this study. The predicted sites involved in PLD2 binding to CRMP-2 are shown schematically as a gray column. 2 µg of GST, C-terminal deletion mutants of CRMP-2 (B) or the N-terminal deletion mutants (C) were incubated with PLD2 (0.1 µg) and examined by sequential pull-down assay, as described under “Experimental Procedure”. The final pellets were loaded onto a gel for immunoblot analysis. The results shown are representative of two separate experiments.

**Fig. 5. In vivo interaction between CRMP-2 and PLD2.** COS-7 cells were cotransfected with wild-type human PLD2 (hPLD2) and flag-tagged human CRMP-2 (hCRMP-2) as indicated. Cells were lysed and sonicated in extraction buffer containing 1% TX-100 as described under “Experimental Procedures”. After centrifugation, the resulting supernatants (1 mg) were incubated with anti-flag M2 agarose, and immunoblotted using anti PLD-, actin- or flag-specific antibody.
Results were reproducible in two experiments performed with independent preparations.

**Fig. 6. Inhibition of PLD2 by CRMP-2 in vitro.** A PIP$_2$-dependent PLD activity assay was performed in the presence of the indicated amounts of CRMP-2 purified from rat brain (■), GST (□), GST-human CRMP-2 (●) or GST fusion proteins of its N-terminal deleted CRMP-2 (N3(243-573) (▲), N4(301-573) (△)) purified from *E. coli* as described under “Experimental Procedures”. Results are expressed as a percentage of the control (basal PLD2-induced choline release). The results shown are the means ± S.D of two different experiments performed in duplicate.

**Fig. 7. The effect of CRMP-2 on PLD2 activity in PC12 cells.** *A.* PC12 cells grown in the absence (-) or presence (+) of tetracycline (Tet) were transfected with 0.5 µg of cDNAs of an empty vector (Vector) or of a flag-tagged human CRMP-2 (CRMP-2), prelabeled with [$^{3}$H]myristate, and PLD activity was determined by transphosphatidylation, as described under “Experimental Procedure”. The results were reproducible in two experiments performed with independent preparations. *B.* After the transient expression of vector or flag-tagged human CRMP-2 in PC12 cells, the total lysates (20 µg) were loaded onto a gel and then performed immunoblot analysis using anti-PLD, flag, or actin antibody. The results shown are representative of two separate experiments.

**Fig. 8. Semaphorin 3A-dependent inhibition of PLD2 in PC12 cells.** PC12 cells cultured in the absence (-) or presence (+) of tetracycline (Tet) were treated with the indicated amount of Semaphorin 3A for 5 min and the PLD activity measured, as described under “Experimental Procedure”. The existence of neuropilin-1 was detected in PC12 cells cultured in the same condition by immunoblot analysis (*inset*). One unit of Semaphorin 3A was defined as the amount required in 1 ml of culture to collapse 50% of the growth cones of chick dorsal root ganglion neurons. The results shown are representative of two separate experiments.

**Fig. 9. The co-localization of PLD2 and CRMP-2 in differentiated PC12 cells.** *A.* PLD2-
overexpressing PC12 cells were cultured in 100 ng/ml of NGF containing Dulbecco’s modified Eagle’s medium in the absence (-) or presence (+) of tetracycline (Tet) for 4 days. PLD2 in the PC12 cells was stained using anti-PLD antibody, as described under “Experimental Procedures”. B. PLD2-overexpressing PC12 cells were transfected with flag-tagged hCRMP-2 and then cultured in 100 ng/ml of NGF containing Dulbecco’s modified Eagle’s medium in the absence of tetracycline for 4 days. The localization of PLD2 and CRMP-2 was respectively detected by anti-PLD and -flag antibody as described under “Experimental Procedures”. The *arrowheads* point to regions of PLD2 and CRMP-2 co-localization in the distal tips of neurites. The results were reproducible in two experiments performed with independent preparations.
Fig. 1

A. Hydroxyapatite column chromatography

B. Coomassie-staining

C. Western blotting

- PLD2

+ PLD2

p62
A

| Protein  | Sequence                                      |
|----------|-----------------------------------------------|
| CRMP-1   | MSHQGKKSIP HITSDRLLIR GGR1NDDQS GYADVYLEDG LIKQIGENLI |
| CRMP-2   | MSYQGKKNIP RITSDRLLIK GGK1VNDDQS FYADIYMEDG LIKQIGENLI |
| CRMP-3   | IPR1TSDRLLL IKGGKIVNDD QSFHADLYVE DGLIKQIGEN L1VPGGIKTI |
| CRMP-4   | MSYQGKKNIP RITSDRLLIK GGR1VNDDGS FYADIYMEDG LIKQIGDNLI |
| p62      | KKNIP RITSDLLL1K |

B

Blot: Anti-CRMP-2 ab.

Fig. 2
A

Purified CRMP-2: + +
PLD2: - +

IP: Anti-PLD ab.

---

B

| 1  | 65 | 192 | 201 | 310 | 314 | 476 | 613 | 724 | 826 | 934 |
|----|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|    |    | PX  | PH  | I   | II  | III | IV  | CT  |     |     |
| F1 | F2 | F3  | F4  | F5  | F6  |     |     |     |     |     |

---

C

GST F1 F2 F3 F4 F5 F6

Blot: Anti-PLD ab.

---

Fig. 3
Fig. 3

Blot: Anti-PLD ab.

D

GST F1 PX PH

CRMP-2
Fig. 4

A

| Protein | Start-End |
|---------|-----------|
| WT (1-573) | 1-573 |
| C1 (1-450) | 143-178 |
| C2 (1-300) | 243-300 |
| C3 (1-143) | 300-369 |
| N1 (144-573) | 143-178 |
| N2 (178-573) | 178-243 |
| N3 (243-573) | 243-300 |
| N4 (301-573) | 300-369 |
| N5 (334-573) | 334-573 |
| N6 (369-573) | 369-573 |

B

Blot: Anti-PLD ab.

GST  WT  C1  C2  C3

Ponceau staining

Fig. 4
Fig. 4
Fig. 5

- hPLD2: - + - +
- hCRMP-2: - - + +

| Total lysate | IP: Anti-flag ab. |
|--------------|-------------------|
| PLD2         |                   |
| CRMP-2       |                   |
| Actin        |                   |
| PLD2         |                   |
| CRMP-2       |                   |
Fig. 6
A Vector CRMP-2

PBtOH formation [% of Total lipids]

Tet: + - + -

0 1 2 3

B Tet: + - + -

Vector CRMP-2

PLD2 CRMP-2 Actin

Fig. 7
Fig. 8

PBtOH formation [% of Total lipids]

Semaphorin 3A (Unit/ml): 0 0.1 1 10

[Inset: Tet + Pcp, band labeled Neuropilin-1]
Fig. 9

A

Tet: +   -

Flag -CRMP-2

B

PLD2

Merged

Fig. 9
Collapsin response mediator protein-2 inhibits neuronal phospholipase D2 activity by direct interaction
Sukmook Lee, Jung Hwan Kim, Chang Sup Lee, Jong Hyun Kim, Youndong Kim, Kyun Heo, Yasuo Ihara, Yoshio Goshima, Pann-Ghill Suh and Sung Ho Ryu

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