Munc-18-1 Inhibits Phospholipase D Activity by Direct Interaction in an Epidermal Growth Factor-reversible Manner*‡

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Mammalian phospholipase D (PLD) has been reported to be a key enzyme for epidermal growth factor (EGF)-induced cellular signaling, however, the regulatory mechanism of PLD is still unclear. In this report, we found that Munc-18-1 is a potent negative regulator of PLD in the basal state and that its inhibition is abolished by EGF stimulation. We investigated PLD-binding proteins obtained from rat brain extract, and identified a 67-kDa protein as Munc-18-1 by peptide-mass fingerprinting. The direct association between PLD and Munc-18-1 was confirmed by in vitro binding analysis using the purified proteins, and their binding sites were identified as the phox homology domain of PLD and multiple sites of Munc-18-1. PLD activity was potently inhibited by Munc-18-1 in vitro (IC_{50} = 2–5 nM), and the cotransfection of COS-7 cells with Munc-18-1 and PLD inhibited basal PLD activity in vivo. In the basal state, Munc-18-1 coprecipitated with PLD and colocalized with PLD2 at the plasma membrane of COS-7 cells. EGF treatment triggered the dissociation of Munc-18-1 from PLD when PLD was activated by EGF. The dissociation of the endogenous interaction between Munc-18-1 and PLD, and the activation of PLD by EGF were also observed in primary cultured chromaffin cells. These results suggest that Munc-18-1 is a potent negative regulator of basal PLD activity and that EGF stimulation abolishes this interaction.

Mammalian phospholipase D (PLD) is a membrane-bound enzyme that hydrolyzes phosphatidylcholine to generate a multifunctional lipid, phosphatidic acid (PA), in response to a variety of signals, including hormones, neurotransmitters, and growth factors (1). PA itself has been shown to be an intracellular lipid second messenger and to be involved in multiple growth factors (1). PA itself has been shown to be an intracellular lipid second messenger and to be involved in multiple growth factors (1). However, the regulatory mechanisms of PLD activity in its various functions have not been well characterized. Therefore, the identification of novel proteins that interact with PLD may provide clues about its function, not only in terms of the mechanisms that regulate PLD, but also in terms of the functional association between these regulators and specific cellular events.

Many reports have suggested that PLD plays a role in the transduction of the intracellular signals initiated by various agonists (1, 12). Because the product of PLD, PA, is involved in many cellular signaling events, agonist-induced PLD activation must be tightly controlled. Moreover, PLD is known to be positively regulated by various proteins and signals, for example, by ADP-ribosylation factor (ARF), protein kinase C, and EGF (12–13). In particular, EGF stimulation increases PLD1 activity and this activation is downstream of PIP_2 hydrolyzing phospholipase C and subsequent protein kinase C activation (14). However, the molecular mechanism of PLD2 activation by EGF is at present unclear, even though a recent report suggested that ARF4 is a potential mediator (15). Moreover, αβ-synuclein (16), α-actinin (17), actin (18), and collapsin response mediator protein-2 (19) have been reported to inhibit basal PLD activity, but the nature of the signal-dependent modulation of PLD activity by its negative regulator after agonist stimulation remains unknown.

Munc-18-1 is a 67-kDa protein, which was originally identified as a major brain protein, which binds to syntaxin, a synaptic vesicle fusion protein (20–22). Mammals express three highly homologous isoforms of Munc-18 (23). Munc-18-1 is enriched in neurons, whereas Munc-18-2 and Munc-18-3 are expressed ubiquitously (23). Knockouts of Munc-18-1 have demonstrated that this protein is essential for synaptic vesicle exocytosis (24, 25), but the molecular mode of action of Munc-18-1 is not known in detail. Even though Munc-18-1 is an important protein in neuronal exocytosis and its protein-protein interactions may be critical in the fusion event, relatively few of its binding partners have been identified (26–28). Furthermore, it has not been previously reported that Munc-18-1 can dynamically inhibit enzyme activity by direct interaction or that it is involved in cellular signaling events.

In this study, we found for the first time that Munc-18-1 is a PLD-interacting molecule, and its inhibitory effect on PLD occurs by direct interaction with the PX domain of PLD. Fur-
thermore, we found that this negative regulation of PLD activity by Munc-18-1 can be overcome by EGF stimulation, which thus offers a novel mechanism for PLD regulation.

**EXPERIMENTAL PROCEDURES**

**Materials**—The enhanced chemiluminescence (ECL) kit, dipalmitoylphosphatidyl-1-myristyl-2-

1. Chloroform, chelating Sepharose, and glutathione-Sepharose 4B were purchased from Amersham Biosciences; [H]myristic acid from PerkinElmer Life Sciences; Silica Gel 60 thin-layer chromatography plates from MERCK (Darmstadt, Germany); Protein A-Sepharose from RepliGen (Needham, MA); Dulbecco’s modified Eagle’s medium and LipofectAMINE from Invitrogen (Carlsbad, CA); ovalfsh calf serum from HyClone (Logan, UT); EGF from the Daewoong Pharmaceutical Co. (Seoul, Republic of Korea); cholic acid from U. S. Biochemical Corp. (Cleveland, OH); horseradish peroxidase-conjugated goat anti-rabbit IgG and anti-mouse IgG, IgM, and IgA from Kirkegaard and Perry Laboratories, Inc. (Gaithersburg, MD); and monoclonal anti-Munc-18 antibody from BD Transduction Laboratories, (Franklin Lakes, NJ). Polyclonal antibody (mSTP4) recognizing both PLD1 and PLD2 was produced as described previously (29). All other chemicals were purchased from Sigma.

**Coimmunoprecipitation Analysis of PLD from Rat Brain Extract**—Rat brains (3 g) were lysed by homogenization in buffer A (20 mM Tris-HCl, pH 7.0, 150 mM NaCl, 1 mM MgCl₂, 1 mM EGTA, 1% Triton X-100, 1% cholic acid). Lysates were centrifuged at 100,000 g at 4 °C, and the supernatants were incubated with anti-PLD antibody immobilized to Protein A-Sepharose beads. The precipitates were washed four times and subjected to SDS-PAGE followed by immunoblot analysis.

**Identification of Protein by Peptide Mass Fingerprinting Using Matrix-assisted Laser Desorption Ionization (MALDI) Time-of-flight Mass Spectrometry**—After silver staining, candidate bands were excised from the gel and digested with trypsin. A 1-μl aliquot of the total 30 μl of digest was used for peptide mass fingerprinting. Masses of the tryptic peptides were measured using a Bruker Reflex III mass spectrometer, as previously described (17, 30). MALDI was performed using α-cyano-4-hydroxycinnamic acid as the matrix. Trypsin autolysis products were used for internal calibration. Delayed ion extraction resulted in peptide masses with a mass accuracy of better than 0.1 Da on average. Mass values were compared against the NCBI data base using Profound (31).

**Construction and Preparation of Glutathione S-Transferase (GST) Fusion, FLAG- or His-tagged Proteins**—The full-length cDNAs of rat PLD1 or human PLD2 were digested into fragments containing specific regions and ligated into pGEX4T3 vector, as previously reported (18). The full-length cDNA of human PLD2 was deleted (167-206) and ligated into pcDNA 3.1 vector for transfecting into COS-7 cells. Murine Munc-18-1 plasmid in pGEK vector was generously provided by Dr. Thomas C. Sudhof (Howard Hughes Medical Institute, University of Texas Southwestern Medical School, Dallas, TX) (20). A 1.8-kb Munc-18-1 insert was obtained by PCR and also digested into fragments containing specific domains. Domains 1, 2-1, and 3-a were digested with BamHI or EcoRI, domain 2-2 was ligated with EcoRI or XhoI, and domain 3-b with BamHI or XhoI, as previously reported (32). These fragments were ligated into pGEX4T1 vector. The 3.0-kb murine Munc-
18-1 insert was ligated into the NcoI or HindIII site of the pRSETB vector for transformation into *Escherichia coli* to produce His<sub>6</sub>-tagged Munc-18-1, which was purified by chelating-Sepharose affinity column chromatography using 80 mM imidazole as eluant. The 1.8-kb Munc-18-1 insert was digested and ligated into the EcoRI or HindIII sites of the cytomegalovirus vector for transfecting into COS-7 cells, thus resulting in the FLAG tagging of Munc-18-1. GST fusion PX domains of p40<sup>PH</sup>, FISH, and sorting nexin 1 (SNX1) were kindly provided by Dr. Michael B. Yaffe (Massachusetts Institute of Technology, MA) (33), Dr. Peter Lock (The Ludwig Institute for Cancer Research, Royal Melbourne Hospital, Australia) (34), and Dr. Rohan D. Teasdale (Institute for Molecular Bioscience, The University of Queensland, Australia) (35), respectively. Inserts of p40<sup>PH</sup>, FISH, and the SNX1-PX domain were obtained by PCR and digested into fragments containing specific domains by using EcoRI or BamHI, and XhoI or EcoRI. These fragments were then ligated into pGEX4T1 vector. *E. coli* BL21 cells were transformed with individual expression vectors encoding the GST fusion proteins and purified by standard methods (36) that involved: transforming the corresponding intensities of spots obtained in the absence of 1-butanol were measured, and PLD activity was obtained by subtracting the corresponding intensities of spots obtained in the absence of 1-butanol.

**Immunoprecipitation**—Immunoprecipitation was performed as described previously (14). Briefly, cells were lysed with PLD assay buffer (50 mM HEPES/NaOH, pH 7.3, 3 mM EGTA, 3 mM CaCl<sub>2</sub>, 3 mM MgCl<sub>2</sub>, and 80 mM KCl) containing 0.1% Triton X-100 and 0.1% cholic acid at 4 °C for 2 h. The resulting complexes were washed three times with 1 ml of PLD assay buffer containing 0.1% Triton X-100 and 0.1% cholic acid, and once with a buffer without detergent. Samples were then subjected to SDS-PAGE, and immunoblotting using anti-Munc-18 or anti-PLD antibody.

**PLD Activity Assay in Vitro—**PIP<sub>2</sub>-dependent PLD activity was assayed by measuring choline release from phosphatidyethanolamine using dipalmitoylphosphatidylcholine with minor modifications of a previously described method (38). The reaction was carried out for 20 min at 37 °C in 125 μl of assay mixture containing PLD assay buffer (50 mM HEPES/NaOH, pH 7.3, 3 mM EGTA, 3 mM CaCl<sub>2</sub>, 3 mM MgCl<sub>2</sub>, and 80 mM KCl). Oleate-dependent PLD activity was assayed as previously described (29). The reaction mixture (175 μl) containing 50 mM HEPES/NaOH, pH 7.0, 2 mM EGTA, 1.7 mM CaCl<sub>2</sub>, 20 μM sodium oleate, and 0.1 M KCl was incubated at 30 °C for 1 h.

**Cell Culture—**COS-7 cells were maintained in growth medium composed of Dulbecco’s modified Eagle’s medium supplemented with 10% bovine calf serum at 37 °C in a humidified CO<sub>2</sub>-controlled (5%) incubator. COS-7 cells were transfected using LipofectAMINE, as described previously (19, 39). Bovine adrenal chromaffin cells were prepared as described previously (40) and primary cultured in a growth medium composed of Dulbecco’s modified Eagle’s medium/P/F-12 supplemented with 10% bovine calf serum at 37 °C in a humidified CO<sub>2</sub>-controlled (5%) incubator.

**PLD Activity Assay in Cells—**PLD activity was assayed by measuring the formation of P<sub>B</sub>T (39). The intensities of P<sub>B</sub>T spots in the presence of 1-butanol were measured, and PLD activity was obtained by subtracting the corresponding intensities of spots obtained in the absence of 1-butanol.

**Immunoblot Analysis—**Proteins were denatured by boiling for 5 min at 95 °C in Laemmli sample buffer (37), separated by SDS-PAGE, and immunoblotted as described previously (19).
and 80 mM KCl) containing 1% Triton X-100, 1% cholic acid, and 1 mM phenylmethylsulfonyl fluoride. After brief sonication, the cell lysates were centrifuged at 100,000 g for 30 min at 4 °C. The supernatants (1 mg of protein) were then incubated with anti-PLD antibody immobilized on protein A-Sepharose for 6 h at 4 °C. The resulting immune complexes were washed three times with 1 ml of PLD assay buffer containing 1% Triton X-100 and 1% cholic acid and once with a buffer without detergent. Samples were then subjected to SDS-PAGE, and immunoblotting using anti-Munc-18 or anti-PLD antibody.

**RESULTS**

**Munc-18-1 Was Identified as a PLD-interacting Protein in Rat Brain Extract**—Because the regulation of PLD might occur via direct interaction with other binding partners, the enrichment of PLD in rat brain extract led us to perform a coimmunoprecipitation assay to isolate proteins that bind directly with PLD. After the precipitation with anti-PLD antibody from rat brain extract and protein analysis by SDS-PAGE and silver staining, the coprecipitate was found to contain a PLD-binding protein with a relative molecular weight of 67,000 (Fig. 1A). A 67-kDa band in the PLD precipitate was excised from a gel for identification by peptide mass fingerprinting. A peptide mixture of the trypsin-digested p67 was eluted and analyzed by MALDI-TOF mass spectrometry. Fig. 1B shows the peptide mass map of the protein. A search for these masses in a comprehensive sequence data base showed that 6 masses matched the calculated tryptic peptide masses of Munc-18-1 with an accuracy of <0.1 Da (Fig. 1C). These peptides covered 12% of the sequence of Munc-18-1. To further verify the identity of this protein, PLDs were immunoprecipitated from rat brain extract and the presence of Munc-18-1 was confirmed by immunoblotting with anti-Munc-18 antibody. As shown in Fig. 1D, Munc-18-1 was specifically precipitated by anti-PLD antibody but not by preimmune serum.

**Munc-18-1 Interacts Directly with PLD2**—To determine whether Munc-18-1 binds directly to PLD, an *in vitro* binding analysis was performed with the purified proteins. Because PLD2 was precipitated more than PLD1 in the rat brain extract immunoprecipitate, we used purified PLD2 for the *in vitro* binding analysis. As shown in Fig. 1E, the binding of purified Munc-18-1 increased on increasing the amount of PLD2. This result suggests that the coprecipitated Munc-18-1 by PLD antibody from rat brain extract may have been because of direct interaction with PLD2. PLD1 was also found to interact with Munc-18-1 in a similar manner (data not shown).

**The PLD2-PX Domain C-terminal Region Contains the Munc-18-1 Interacting Site**—To determine the regions of PLD2 that interact with Munc-18-1, fragments of PLD2 were generated as GST fusion proteins (Fig. 2A). The ability of the six GST fusion proteins to bind Munc-18-1 was examined by using an *in vitro* binding assay with purified His<sub>s</sub>-Munc-18-1. GST-F<sub>1</sub>-1–314 was found to bind to Munc-18-1 most strongly (Fig. 2B). To more precisely map the F1 region required for Munc-18-1 binding, GST fusion proteins of the F1 region serial deletion constructs, and of the PX, the pleckstrin homology, or the F1 region without the PX domain construct, were generated (Fig. 2A). As shown in Fig. 2C, F1-2-(1–206) interacted with Munc-18-1, but the shorter fragments showed little interaction. The PX domain (66–195) itself also interacted with Munc-18-1 but the pleckstrin homology domain or the F1 region, which do not contain the PX domain, had little bindings with Munc-18-1 (Fig. 2D). These results led us to conclude that the C-terminal region of the PLD2-PX domain (167–195) makes a major contribution to Munc-18-1 binding.

**Multiple Sites of Munc-18-1 Are Involved in the Interaction with PLD2**—To determine the regions of Munc-18-1 that interact with PLD2, fragments of Munc-18-1 were generated as GST fusion proteins (Fig. 3A) considering the crystal structure of Munc-18-1 (32). As shown in Fig. 3B, domain 1 (D1; 4–134), domain 2-2 (D2-2; 480–592), and domain 3-a (D3-a; 246–361) bound to PLD2 but not as well as whole Munc-18-1 bound to PLD2. This result suggests that multiple sites of Munc-18-1 cooperate to produce high affinity binding between Munc-18-1 and PLD2.

**Munc-18-1 Specifically Interacts with the PLD-PX Domain**—To check whether Munc-18-1 binds to the PX domains of other proteins, we tested its interactions with the PX domains of PLD1, PLD2, p40<sub>phox</sub>, FISH, and SNX1. The sequence alignment of the PLD1-, PLD2-, p40<sub>phox</sub>-FISH-, and SNX1-PX domains was performed using the Clustal W software (Fig. 4A). Using a GST pull-down assay, we found that Munc-18-1 does not bind to the PX domains of p40<sub>phox</sub>, FISH, or SNX1, whereas it clearly interacted with both PX domains of PLD1 and PLD2 (Fig. 4B). This result suggests that Munc-18-1 specifically interacts with the PLD-PX domain. According to the sequence alignment result, the homology between the PLD1-PX domain and the PLD2-PX domain is high (46%) and the homology between PLD-PX domains and other PX domains is relatively low (less than 20%). Especially, the homology between the C-terminal 29 amino acids of PLD-PX domains, which correspond to 167–195 of PLD2, is very high (68%), which supports that Munc-18-1 binds to the C-terminal of the PLD-PX domain. To further confirm the importance of the C-terminal of the
PLD-PX domain, we constructed a PLD2 deletion mutant (Δ167–206) according to the result of Fig. 2C and tested the interaction with Munc-18-1 by *in vitro* binding analysis (Fig. 4C) and *in vivo* coimmunoprecipitation analysis (Fig. 4D). As shown in Fig. 4, C and D, the PLD2 deletion mutant (Δ167–206) did not interact with Munc-18 either *in vitro* or *in vivo*, whereas PLD2 wild type showed a normal interaction. Taken together these results and the result from Fig. 2D strongly
PLD simultaneously without saturating the PLD blot; how- it was difficult to show endogenous PLD and overexpressed expression of endogenous PLD in COS-7 cells was very low, and

or PLD2, as described under "Experimental Procedures." Results are expressed as percentages of the control (basal PLD-induced choline release). Open and closed symbols mean PIP2- and oleate-dependent activity assays, respectively. The data shown are the mean ± S.E. from two independent assays.

support that the C-terminal region (167–195) of the PLD2- PX domain is critically important for Munc-18-1 interaction.

Munc-18-1 Inhibits PLD Activity—As Munc-18-1 was found to bind to the PX domain of PLD, and the N-terminal region of PLD has been reported to be important for PLD activity regulation (10), we tested the effect of Munc-18-1 on PLD activity in vitro and in vivo. As shown in Fig. 5, the in vitro activity of PLD was inhibited specifically by His6-Munc-18-1 in a concentration-dependent manner. The concentration required for half-maxi- mal inhibition was determined to be ~5 nM in a PIP2-dependent PLD activity assay. To exclude the possibility that the inhibition might have resulted from the masking of PIP2 by Munc-18-1, we examined the effect of Munc-18-1 on PLD2 activity in an oleate-dependent activity assay. The inhibition of PLD2 activity by Munc-18-1 was found to be unchanged; ~2 nM Munc-18-1 was required to inhibit PLD2 activity by 50% (Fig. 5). To test the effect of Munc-18-1 on in vivo PLD activity, PLD1 or PLD2 and Munc-18-1 were cotransfected into COS-7 cells and the effect of Munc-18-1 on basal PLD activity was determined. As shown in Fig. 6, the transfection of increasing amounts of Munc-18-1 DNA resulted in the inhibition of en- dogenous and overexpressed PLD2 activity. The expression levels of Munc-18-1 and PLD are shown in the lower panel. The expression of endogenous PLD in COS-7 cells was very low, and it was difficult to show endogenous PLD and overexpressed PLD simultaneously without saturating the PLD blot; how- ever, at longer exposure times, it was possible to detect the endogenous PLD (data not shown). Munc-18-1 also inhibited PLD1 activity both in vitro and in vivo. Taken together, these results suggest that Munc-18-1 is a negative regulator of PLD.

EGF Triggers the Dissociation of the EGF-Munc-18-1 Complex and Abolishes Munc-18-1-induced PLD Inhibition—Several reports have suggested that PLD plays a role in the transduction of intracellular signals initiated by EGF (14, 41, 42). Here, we checked the effect of EGF on the PLD-Munc-18-1 interaction and PLD activity. COS-7 cells were cotransfected with PLDs and vector, or with PLDs and Munc-18-1, and the PLD activity was determined by accumulating phosphatidylbutanol for 5 min. Munc-18-1 inhibited PLD activity in the basal state as shown in Fig. 6. However, no difference was observed between the control and the Munc-18-1-transfected cells in terms of PLD activity after treating with EGF (Fig. 7A). Fig. 7, B and C, illustrate the interaction between PLD and Munc-18-1 in the basal state, and the abrogating effect of EGF treatment on this interaction. Furthermore, no re-association of Munc-18-1 and PLD was observed after EGF treatment. Taken together, these results suggest that EGF recovers PLD activity basally inhibited by Munc-18-1. Moreover, PLD acti- vation was found to be related with the abrogation of the PLD-Munc-18-1 interaction.

Munc-18-1 Localizes to the Membrane with PLD2 and Translocates to the Cytosol by EGF Treatment—Immunocytochemical analysis was performed to localize Munc-18-1 and PLD2 in COS-7 cells. The association between PLD2 and Munc-18-1 was confirmed in transfected cells, as shown in Fig. 8A. Munc-18-1 was basally localized in the cytosol in Munc-18-1 single transfected cells and PLD2 was localized to the plasma membrane in PLD2 single transfected cells (Fig. 8B). However, PLD2 and Munc-18-1 colocalized to the plasma membrane in PLD2 and Munc-18-1 cotransfected cells. Moreover, at 0.5, 1, 2, or 5 min after EGF treatment, Munc-18-1 dissociated from PLD2 and moved into the cytosol from the plasma membrane (Fig. 8C). Therefore, we suggest that EGF stimulation modifies the interaction between Munc-18-1 and PLD2 and triggers the translocation of Munc-18-1 from the plasma membrane to the cytosol.

DISCUSSION

PLDs are involved in various signal transduction pathways and have diverse functions. To ensure that they play their intended roles, their activities must be tightly regulated. Never- theless, the signal-dependent regulation of PLDs by their negative regulators is poorly understood. Here we report, for the first time, that Munc-18-1 is a potent negative regulator of PLD and that EGF stimulation dynam- ically regulates the interaction between PLD and Munc-18-1.

EGF Triggers the Dissociation of Munc-18-1 and PLD to Activate PLD in Bovine Adrenal Chromaffin Cells—Primary cultured bovine adrenal chromaffin cells were used to examine the effect of the EGF signal on the endogenous PLD-Munc-18-1 interaction and on PLD activity. PLD activity was determined by accumulating phosphatidylbutanol for 5 min and measuring its level. As shown in Fig. 9, A and B, EGF treatment in bovine adrenal chromaffin cells abolished the endogenous interaction between PLD and Munc-18-1 and increased PLD activity. Taken together, these results suggest that Munc-18-1 is a negative regulator of PLD and that EGF stimulation dynami- cally regulates the interaction between PLD and Munc-18-1.

PA has been reported to be a mitogenic mediator, and many growth stimulatory ligands are known to stimulate PA produc- tion by PLD in various cells (43). Therefore, PLD activity must be tightly controlled by certain negative regulators in the basal cellular state. The currently classified PLD inhibitors are cytoskeleton-related proteins such as, fodrin, α-actinin, actin, and gelsolin (17, 18, 44, 45) or metabolic proteins like aldolase (30). Synaptojanin, clathrin assembly protein 3, and amphiphysin (36, 46, 47) have been reported to be vesicle endocytosis-related proteins that regulate PLD activity. Thus, the results available to date suggest that the diverse and function-related PLD inhibitors may regulate PLD by different mechanisms in different cells. In this work, we found that a vesicle exocytosis-related protein, Munc-18-1 is a negative regulator of PLD. Here, we suggest that various kinds of inhibitors are needed for the signal-specific cellular regulation of PLD.

Several studies have suggested that PLD plays a role in the
FIG. 6. Munc-18-1 inhibits PLD activity in COS-7 cells. COS-7 cells were cotransfected with PLD1 or PLD2 and different amounts of Munc-18-1. After serum starvation for 24 h, the intensities of PBt spots after 5 min accumulation in the presence of 0.4% 1-butanol were measured, and results were obtained by subtracting the corresponding intensities of spots obtained in the absence of 1-butanol, as described under “Experimental Procedures.” The data shown are the mean ± S.E. from two independent assays. The expression levels of PLDs and Munc-18-1 are shown in the boxes. Arrows (—) indicate molecular masses (kDa) of standard proteins.

FIG. 7. EGF triggers the dissociation of the PLD-Munc-18-1 complex and thereby relieves the inhibition of PLD activity. A, COS-7 cells were cotransfected with PLDs and vector, or with PLDs and Munc-18-1. After serum starvation for 24 h, EGF stimulation (100 nM) was performed for 0 or 5 min. The intensities of PBt spots after 5 min accumulation in the presence of 0.4% 1-butanol were measured, and results were obtained by subtracting the corresponding intensities of spots obtained in the absence of 1-butanol, as described under “Experimental Procedures.” The data shown are the mean ± S.E. from two independent assays. B, COS-7 cells were cotransfected with PLD2 and vector, or with PLD2 and Munc-18-1. After serum starvation for 24 h, EGF stimulation was performed for 0, 0.5, 1, 2, or 5 min. Cells were lysed with ice-cold PLD buffer, as described under “Experimental Procedures.” Total cell lysates (TOTAL) and immune complexes (I.P.) with anti-PLD antibody were subjected to SDS-PAGE and followed by immunoblot analysis. Tubulin was blotted as a control. The data shown are representative of two independent experiments. C, COS-7 cells were cotransfected with PLD1 and vector, or with PLD1 and Munc-18-1. After serum starvation for 24 h, EGF stimulation was performed for 0, 0.5, 1, 2, or 5 min. Cells were then lysed with ice-cold PLD buffer, as described under “Experimental Procedures.” Total cell lysates (TOTAL) and immune complexes (I.P.) with anti-PLD antibody were subjected to SDS-PAGE and immunoblotted. Tubulin was blotted as a control. The data shown are representative of two independent experiments. Arrows (—) in B and C indicate molecular masses (kDa) of standard proteins.
transduction of intracellular signals initiated by EGF (14, 41, 42). PLD activation by EGF occurs downstream of PIP2 hydrolyzing phospholipase C, and this activation is known to be dependent upon subsequent protein kinase C activation, which phosphorylates PLD1 (14). EGF was also found to cause the tyrosine phosphorylation of PLD2, although this phosho-

**FIG. 8.** Munc-18-1 localizes to the membrane with PLD2 but is translocated into the cytosol after EGF treatment. A, COS-7 cells were cotransfected with vector and Munc-18-1, or with PLD2 and Munc-18-1. Cells were lysed with ice-cold PLD buffer, as described under “Experimental Procedures.” Total cell lysates (TOTAL) and immune complexes (I.P.) with anti-PLD antibody were subjected to SDS-PAGE and immunoblotted. Tubulin was blotted as a control. The data shown are representative of two independent experiments. Arrows (←) indicate molecular masses (kDa) of standard proteins. B, COS-7 cells were transfected with Munc-18-1 only or with PLD2 only. To observe the localizations of Munc-18-1 and PLD2 in COS-7 cells, immunocytochemical analysis using polyclonal anti-PLD antibody or monoclonal anti-Munc-18 antibody was performed, as described under “Experimental Procedures.” Fluorescein isothiocyanate-conjugated anti-mouse antibody (left) and rhodamine-conjugated anti-rabbit antibody (right) were observed under a confocal microscope. The data shown are representative of three independent experiments. C, COS-7 cells were cotransfected with PLD2 and Munc-18-1. After serum starvation for 24 h, cells were stimulated with EGF for 0, 0.5, 1, 2, or 5 min. Immunocytochemical analysis was then performed using polyclonal anti-PLD antibody or monoclonal anti-Munc-18 antibody to determine the localizations of Munc-18-1 and PLD2 in COS-7 cells, as described under “Experimental Procedures.” The fluorescein isothiocyanate-conjugated anti-mouse antibody image (left), the rhodamine-conjugated anti-rabbit antibody image (middle), and the merged image (right) were obtained by confocal microscope. The data shown are representative of five independent experiments.

**FIG. 9.** EGF triggers the dissociation of Munc-18-1 and PLD to activate PLD in bovine adrenal chromaffin cells. A, bovine chromaffin cells were primary cultured and after serum starvation for 24 h, the cells were treated with EGF for 0 or 5 min. The intensities of PBT spots after 5 min accumulation in the presence of 0.4% 1-butanol were measured, and results were obtained by subtracting the corresponding intensities of spots obtained in the absence of 1-butanol, as described under “Experimental Procedures.” The data shown are the mean ± S.E. from two independent assays. B, bovine chromaffin cells were primary cultured, and after serum starvation for 24 h, EGF was treated for 0 or 5 min. Cells were lysed with ice-cold PLD buffer, as described under “Experimental Procedures.” Total cell lysates (TOTAL) and immune complexes (I.P.) with anti-PLD antibody were subjected to SDS-PAGE and immunoblotted. Tubulin was blotted as a control. The data shown are representative of two independent experiments. Arrows (←) indicate molecular masses (kDa) of standard proteins.
rylation was not critical for PLD activation (48). Although we cannot explain the mechanism for the dissociation of Munc-18-1 from PLD and the activation of PLD by EGF, one recent report suggested that ARF4 mediates EGF receptor-dependent PLD2 activation (15). In the present study, the abrogation of PLD inhibition and the dissociation of them were triggered by EGF, and this suggests that their interaction can be abolished by EGF receptor stimulation. Furthermore, we checked the effect of ARF1 on the Munc-18-1-induced inhibition of PLD activity in vitro and found that ARF1 abolished the inhibitory effect of Munc-18-1 (data not shown). Thus, we suggest that in the case of the EGF-dependent dissociation of PLD-Munc-18-1, ARF is a candidate modulator.

PLD is involved in protein-protein interactions, and these interactions might be dependent upon a certain domain or motif. In a previous report (17), α-actinin was found to inhibit PLD2 activity by interacting directly with the N-terminal 185 amino acids, which contain the PX domain. In the present study, we confirmed that Munc-18-1 binds with the PLD-PX domain, which means that Munc-18-1 regulates PLD activity by binding to this domain. However, Munc-18-1 was unable to bind to the PX domains of p40phox, FISH, or SNX1, which indicates that the interaction between the PLD-PX and Munc-18-1 is specific. However, the PX domains of PLD1 and PLD2 both bound to Munc-18-1, which means that Munc-18-1 may regulate PLD1 and PLD2 in a similar manner. We also confirmed that the PX domain of PLD2 is not sticky or nonspecific in terms of its interaction with Munc-18-1 (Supplemental Materials Fig. 1). Several PX domains of: cytokine-independent survival kinase, Vam7, SNX3, p40phox, and p47phox are known to be critical sites for phosphoinositide binding (33, 49–52).

Moreover, a recent report suggested that the PX domain of p47phox is involved in intramolecular interactions with the SH3 domain (53). Here, we suggest that the PX domain of PLD is a critical site for interactions with regulators like Munc-18-1, and for the inhibition of PLD activity. Structurally the PX domain has phosphoinositide and SH3 binding sites (54); however, the binding sites for Munc-18-1 in the PLD-PX domain, namely, the C-terminal of the PX domain, is not located at either of these two sites. This indicates that Munc-18-1 binds to the PX surface in a different manner, which suggests that the PX domain may be a multiple binding module for phosphoinositides and proteins. We previously reported that PLC-γ1 also binds to the PX domain of PLD2 and that their interaction is EGF-stimulation dependent (68). Before EGF stimulation, PLC-γ1 and PLD2 showed no interaction, whereas EGF stimulation induced their association in vitro. EGF stimulation was also found to affect the interaction between Munc-18-1 and PLD2 in vivo but in this case, EGF stimulation dissociated Munc-18-1 and PLD2 (Fig. 7). We tested whether PLC-γ1 and Munc-18-1 can simultaneously bind to PLD2 in vitro. Binding of Munc-18-1 to PLD2 decreased the interaction between PLC-γ1 and PLD2 (Supplemental Materials Fig. 2) and this result indicates that PLC-γ1 and Munc-18-1 cannot bind to PLD2 simultaneously. These results suggest that the PX domain of PLD2 may bind to its partners sequentially and perform a multifunctional role.

We also found that multiple sites of Munc-18-1 are essential for interaction with PLD2 and that each domain has a lower affinity for PLD2 than the whole of Munc-18-1. As shown in Fig. 3B, PLD2 did not bind to GST but it bound to whole Munc-18-1 and three of its fragments (D1, D2-2, and D3-a). Misura et al. (32) previously reported that syntaxin 1A binds tightly to Munc-18-1, and elucidated their co-crystal structure. Based on this structure, they found that domain 1 (D1) and domain 3-a (D3-a) of Munc-18-1 and the two amino acids of syntaxin 1A are important for their interaction. In addition, they found that domains 1 (D1), 2-2 (D2-2), and 3-a (D3-a) of Munc-18-1 form a binding pocket for partner proteins. We found that the 40 amino acids (167–206) of PLD2 and three fragments (D1, D2-2, and D3-a), which form a pocket structure in a whole protein, of Munc-18-1 are important for the interaction between PLD2 and Munc-18-1. Syntaxin 1 and double C2 protein (DOC2) also bind to Munc-18-1 at multiple sites (26, 32). Comparisons of the interaction topologies show that it is possible that PLD2 competes with syntaxin 1 for Munc-18-1 binding. Taken together, we suggest that the PLD2 binding sites of Munc-18-1 may form a pocket-like structure and cooperate with each other to bind PLD2.

In primary cultured bovine adrenal chromaffin cells, we detected endogenous PLDs and Munc-18-1. To confirm the results obtained in COS-7 cells, we tested the interaction between PLD and Munc-18-1, and PLD activity in chromaffin cells. We observed that endogenous Munc-18-1 existed in the PLD immune complex in the absence of EGF, and that PLD activity was increased by EGF stimulation and that the PLD-Munc-18-1 interaction was abolished, as in COS-7 cells. This result suggests that the observed relations may be applicable generally to primary cells. EGF is known to influence the growth, migration, differentiation, and maintenance of the central nervous system (55, 56) and to have neuromodulatory actions, as it affects the neuroendocrine system, and suppresses food intake and gastric acid secretion (57–60). Acute and chronic pathological processes, e.g., various cancers, stimulate the production and release of EGF in various cell systems (56, 61). Monitoring studies of EGF in the central nervous system suggest that EGF may participate in several neurological manifestations (e.g., appetite suppression and neuroendocrine alterations), which frequently accompany acute and chronic disease (56). The activation of EGF receptors leads to the glial release of prostaglandin E2, which then acts directly on neurons to stimulate LHRH release (58). PLD1 has been reported to be a key factor of the exocytotic machinery operating in chromaffin cells (62), and in terms of the regulated secretion, ARF6-regulated PLD was reported to be an essential factor in the late stages of exocytosis (63, 64). Although we were unable to check the effect of EGF on the above functions, it would be interesting to study the role of EGF-dependent PLD activation in these cells.

Munc-18-1 is an important protein in neuronal exocytosis and protein-protein interactions may be critical in the fusion event, but only a few binding partners have been identified and the molecular mode of action of Munc-18-1 has not been elucidated. It was previously found that syntaxin 1 is a tight binding partner of Munc-18-1, and this interaction occurs at the plasma membrane (65). In the present work, we observed that Munc-18-1 was mainly localized in the cytosol (Fig. 8B). However, when COS-7 cells were cotransfected with PLD2 and Munc-18-1, most of Munc-18-1 was detected in the plasma membrane region (Fig. 8C) where PLD2 is located. Both syntaxin 1 and PLD2 localize at the plasma membrane and colocalize with Munc-18-1 in the basal state, which suggests that the interaction between these proteins and Munc-18-1 may proceed via a similar mechanism. EGF treatment triggered the translocation of Munc-18-1 from the plasma membrane to the cytosol, and concomitantly, the interaction between PLD and Munc-18-1 disappeared (Fig. 8C). This is the first study to report that Munc-18-1 can inhibit the activity of an enzyme through direct interaction. Summarizing, the exocytosis regulator, Munc-18-1, was found to be affected by EGF signaling. PLD has also been reported to be involved in exocytosis processes, for example, in vesicle trafficking and neurotransmitter release (66, 67). The relationships between EGF signaling and exocytosis, and
the involvement of Munc-18-1-PLD interaction remain interesting topics for future study.

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