Phospholipase D2 Directly Interacts with Aldolase via Its PH Domain†

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ABSTRACT: Mammalian phospholipase D (PLD) has been implicated in the cellular signal transduction pathways leading to diverse physiological events and known to be regulated by many cellular factors. To identify the proteins that interact with PLD, we performed a protein overlay assay with fractions obtained from the sequential column chromatographic separation of rat brain cytosol using purified PLD2 as a probe. A protein of molecular mass 40 kDa, which was detected by anti-PLD antibody with overlaying of the purified PLD2, is shown to be aldolase C by peptide-mass fingerprinting with matrix-assisted laser desorption/ionization-time-of flight mass spectrometry (MALDI-TOF-MS). Aldolase A also showed similar binding properties as aldolase C and was co-immunoprecipitated with PLD2 in COS-7 cells overexpressing PLD2 and aldolase A. The PH domain corresponding to amino acids 201−310 of PLD2 was necessary for the interaction observed in vitro, and aldolase A was found to interact with the PH domain of PLD2 specifically, but not with other PH domains. PLD2 activity was inhibited by the presence of purified aldolase A in a dose-dependent manner, and the inhibition by 50% was observed by the addition of less than micromolar aldolase A. Moreover, the inclusion of the aldolase metabolites fructose 1,6-bisphosphate (F-1,6-P) or glyceraldehyde 3-phosphate (G-3-P) resulted in an enhanced interaction between PLD2 and aldolase A with a concomitant increase in the potential ability of aldolase A to inhibit PLD2, which suggests the existence of a possible regulation of the interaction by the change of intracellular concentrations of glycolytic metabolites.

The hydrolysis of phosphatidylcholine (PC)[1] by mammalian phospholipase D (PLD) to yield choline and phosphatidic acid (PA) has been observed in a wide range of cells after stimulation with a variety of stimuli, including hormones, growth factors, neurotransmitters, and cytokines (*1*−*5*). The intensive research on PLD performed during the past decade has provided an accumulation of evidence for the involvement of PLD in many physiological processes, such as activation of the respiratory burst in neutrophils (*6*), fMLP-induced p38 activation in differentiated HL60 cells (*7*), regulation of the actin cytoskeleton and membrane ruffling (*8*), glucose transport (*9*), and secretion (*10*).

Two forms of PLD isozymes, PLD1 and PLD2 that share approximately 50% amino acid sequence identity, have been characterized to date. PLD1 displays low basal activity and can be activated by monomeric GTP-binding proteins of the ARF and Rho families and by protein kinase C in vitro (*11*, *12*). In contrast, PLD2 has higher specific activity in vitro than PLD1 and appears to be less sensitive to the presence of the known activators of PLD1 (*13*).

Several PLD2 inhibitors have been reported; these include cytoskeleton-related proteins such as fodrin, α-actinin, and gelsolin (*14*−*16*) and vesicle trafficking-related proteins such as amphiphysin, α/β-synuclein, and synaptojanin (*17*−*19*). The stimulation of PLD2 activity is attributed to the deregulation from its molecular constraints. However, the molecular mechanism underlying agonist-dependent activation of PLD2 in intact cells has not yet been elucidated.

To provide a clue concerning the molecular understanding of the regulatory mechanisms of PLD2, the presence of novel PLD2-interacting proteins has been surveyed in the cytosolic fractions of brain. To the best of our knowledge, we found, for the first time, that aldolase A is an inhibitor of PLD2 and binds directly to the PH domain of PLD2 in the presence of the metabolites fructose 1,6-bisphosphate (F-1,6-P) and glyceraldehyde 3-phosphate (G-3-P). This finding suggests that aldolase A, one of the glycolytic enzymes, may regulate

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[1] Abbreviations: PLD, phospholipase D; MALDI-TOF-MS, matrix-assisted laser desorption/ionization-time-of flight mass spectrometry; PH domain, pleckstrin homology domain; PX domain, phox domain; F-1,6-P, fructose 1,6-bisphosphate; G-3-P, glyceraldehyde 3-phosphate; F-1-P, fructose 1-phosphate; DHAP, dihydroxyacetone phosphate; PC, phosphatidylcholine; PA, phosphatidic acid; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-triphosphate; sf9, *Spodoptera frugiperda*; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; GLUT4, glucose transporter 4.

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PLD2 activity depending on the availability of its metabolites.

EXPERIMENTAL PROCEDURES

Materials. The enhanced chemiluminescence kit (ECL system), dipalmitoylphosphatidyl[*methyl*-^{3}H]choline, glutathione Sepharose 4B, chelating-Sepharose, Q-Sepharose and Phenyl Sepharose, and Hi-Trap Heparin column were purchased from Amersham Pharmacia Biotech. Dipalmitoylphosphatidylcholine, dioleoylphosphatidylethanolamine, aldolase diagnostic kits, and glyceraldehyde 3-phosphate were from Sigma. Anti-aldolase antibody was obtained from Chemicon International Inc., horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgA, IgM, and IgG were from Kirkegaard & Perry Laboratories (Gaithersburg, MD), and rabbit anti-goat IgG was from Santa Cruz Biotechnologies Inc. Immobilized protein A was from Pierce, β-octylglucopyranoside and phosphatidylinositol 4,5-bisphosphate (PIP_2) were from Calbiochem (San Diego, CA), and Dulbecco's modified Eagle's medium was from Life Technologies, Inc. A polyclonal antibody that recognizes PLD1 and PLD2 was generated as previously described (*16*). The cDNA of aldolase A was kindly provided by Dr. Jungchul Kim (Kyungpook National University, Korea).

Purification of Recombinant PLD from sf9 Cells. Hexahistidine (His_6)-tagged PLD was expressed in sf9 cells and purified by Chelating Sepharose affinity column chromatography as described previously (*20*).

Fractionation 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 homogenation buffer containing 20 mM Tris, pH 7.6, 1 mM $MgCl_2$, 1 mM PMSF, and 0.1 mM DTT. The homogenate was centrifuged at 100000*g* for 1 h and the resulting supernatant (the cytosolic fraction) collected.

Preparation of the PLD2-Interacting Protein. The cytosolic fraction (900 mg) was applied to a Q-Sepharose anion exchange column (13 cm × 3 cm) equilibrated with buffer A (20 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM $MgCl_2$, and 0.1 mM DTT). Unbound proteins (flow-through fractions) were collected, and NaCl was added to 2 M by adding solid salt. After centrifugation (50000*g*, 20 min), the resulting supernatant was loaded onto a Phenyl Sepharose column (70 cm × 2 cm). Proteins were eluted at a flow rate of 2 mL/min by applying a decreasing gradient of NaCl (from 2 to 0 M) over a period of 60 min. Fractions were collected and tested by blot overlay assay with purified PLD2. Peak fractions were pooled, and diluted with buffer A to adjust the salt concentration to 50 mM NaCl, and then loaded onto a Hi-Trap heparin column (1 mL, Pharmacia) equilibrated with buffer A containing 50 mM NaCl. Bound proteins were then 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 tested by blot overlay assay. The 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 eluted at a flow rate of 0.3 mL/min by applying an increasing gradient of 0−0.25 M KH_2PO_4. Then, fractions were collected and tested using the blot overlay assay with purified PLD2.

PLD2 Overlay Assay. PLD2 overlay assay was performed as previously described (*16*). 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 $CaCl_2$, 3 mM $MgCl_2$, 80 mM KC1) containing 0.1 mM DTT and 5% (w/v) skim milk at room temperature and then incubated with the same buffer containing 1 μg/mL purified PLD2 for 3 h at room temperature. The membranes were 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 h. After the washings, the membranes were subsequently incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies for 1 h and developed using an enhanced chemiluminescence kit as described by the manufacturer.

Identification of a 40 kDa Protein Using Peptide-Mass Fingerprinting by Matrix-Assisted Laser Desorption Ionization/Time-of-Flight Mass Spectrometry. The technique used has been previously described (*16*). In brief, the fraction containing the 40 kDa protein (p40) obtained by hydroxyapatite column chromatography was separated by 10% SDS−PAGE, and the band corresponding to p40 was excised and digested with trypsin (Roche Molecular Biochemicals) for 6 h at 37 °C. The masses of the tryptic peptides so obtained were determined using a Bruker REFLEX reflector time-of-flight mass spectrometer (Bruker-Franzen, Bremen, Germany). Delayed ion extraction resulted in peptide masses with better than 50 ppm mass accuracy on average. Using the amino acid sequences and the mass numbers of the tryptic peptides of p40, the Swiss-Prot database was searched for a protein match.

Construction and Preparation of GST Fusion Proteins. GST fusion proteins of PLD2 fragments were constructed as described previously (*21*). To further construct the phox (PX) and the pleckstrin homology (PH) domains, the N-terminal region (amino acids 1−314) of human PLD2 was used as a template. The PLD2 F1-PX and the PH domains were amplified by PCR using the forward primer 5′-CGG AAT TCA TGG TCA CAG CCC AGG TGG TGG G-3′, the reverse primer 5′-ACG CGT CGA CAG TAC TGA CTT CCA GGA ACT CTG TCA TG-3′, the forward primer 5′-CG GAA TTC ACA GAG TTC CTG GAA GTC AGT C-3′, and the reverse primer 5′-ACGC GTC GAC CGC CAG CTC AGT GAT CTC TTG-3′. The PCR products were digested with the restriction enzymes *Eco*RI and *Sal*I and inserted into pGEX-4T1 vector (Amersham Pharmacia Biotech). The cDNA of aldolase A was cloned into the 5′ *Eco*RI and 3′ *Xho*I sites of the same vector. *E. coli BL21* cells were transformed with the individual expression vectors encoding the GST fusion protein, and after harvesting the cells, GST fusion proteins of PLD2 fragments were purified as described previously (*21*). GST−aldolase fusion proteins and GST-PH domains such as the pleckstrin N- or C-terminus, phospholipase C δ1 (PLC δ1), diacylglycerol kinase (DGK), and Akt 1 were purified by standard methods (*22*) using glutathione−Sepharose 4B (Amersham Pharmacia Biotech).

In Vitro Binding Analysis. In vitro binding between all the GST fusion proteins and aldolase was performed in PLD assay buffer (50 mM HEPES/NaOH, pH 7.3, 3 mM EGTA, 3 mM $CaCl_2$, 3 mM $MgCl_2$, 80 mM KCl) containing 1%

Triton X-100 for 1 h at 4 °C. After a brief centrifugation, the precipitated complexes were washed 3 times in the same buffer before being loaded onto a polyacrylamide gel.

Cell Culture. COS-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) bovine calf serum at 37 °C in a humidified, CO_2-controlled (5%) incubator. For transfection and the transient expression of aldolase and PLD2, COS-7 cells were plated at a density of 1×10^6 cells/well in 100 mm dishes and transfected using LipofectAmine (Life Technologies, Gaithersburg, MD) as previously described (*23*).

Co-immunoprecipitation. COS-7 cells were transfected in combination with pCDNA 3.1 vector harboring human PLD2 and pCMV vector containing aldolase A. The cultured cells were harvested and lysed with PLD assay buffer containing 1% Triton X-100, 1% cholate, and 1 mM PMSF. After a brief sonication, the lysates were centrifuged at 100000*g* for 30 min and the cell extracts (1 mg) incubated respectively with anti-PLD or anit-flag antibody-conjugated protein A Sepharose overnight. After a brief centrifugation, the co-immunoprecipitated complexes were washed 3 times with the same buffer before being loaded onto a gel.

Immunoblot Analysis. Immunoblot analysis was performed as previously reported (*24*). In brief, proteins denatured by boiling for 5 min at 95 °C in Laemmli sample buffer were separated by SDS−PAGE, and transferred to nitrocellulose membranes by electroblotting using the BIO-RAD wet transfer system. After blocking in TTBS buffer (10 mM Tris/HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) containing 5% skimmed milk powder, the membranes were incubated with individual monoclonal or polyclonal antibodies, and this was subsequently followed by another incubation with either anti-mouse or anti-rabbit IgG, as required, coupled to horseradish peroxidase. Detection was performed using an enhanced chemiluminescence kit according to the manufacturers' instructions.

PLD Activity Assay. PLD activity was assayed by measuring choline release from phosphatidylcholine as described previously (*25*) 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, phosphatidylinositol 4,5-bisphosphate, and dipalmitoylphosphatidylcholine in a molar ratio of 16:1.4:1 and dipalmitoylphosphatidyl[*methyl*-^{3}H]choline (total 150 000 cpm/assay). The reaction was terminated by adding 0.3 mL of 1 N HCl/5 mM EGTA and 1 mL of chloroform/methanol/HCl (50:50:0.3). After a brief centrifugation, the [*methyl*-^{3}H]choline in 0.5 mL of the aqueous phase was quantified by liquid scintillation counting.

RESULTS

40 kDa Protein from Rat Brain Was Detected as a PLD2-Direct Binder Using Blot Overlay Assay. The cytosolic fraction (900 mg of proteins) of rat brain was fractionated using sequential column chromatographies, and the fractions obtained were subjected to blot overlay assay to explore PLD2-binding proteins. The protein with an apparent molecular mass of 40 kDa (p40) was detected as a PLD2-binding protein by analyzing the fractions eluted at a linear gradient of 1.8−1.6 M NaCl from a Phenyl Sepharose column (data not shown). p40 eluted from a Hi-Trap heparin column in 0.2 M NaCl and was also reproducibly detected in the blot overlay assay (data not shown). In the final hydroxyapatite column chromatography, p40 was eluted by 0.15−0.2 M KH_2PO_4 (Figure 1A), and the blot overlay assay gave the intensive staining of p40 (Figure 1B). Based on the band intensity of p40 observed by CBB staining, the yield of p40 was assumed to be about 1 mg (data not shown). From these results, p40 was isolated from rat brain as a PLD2-direct binder.

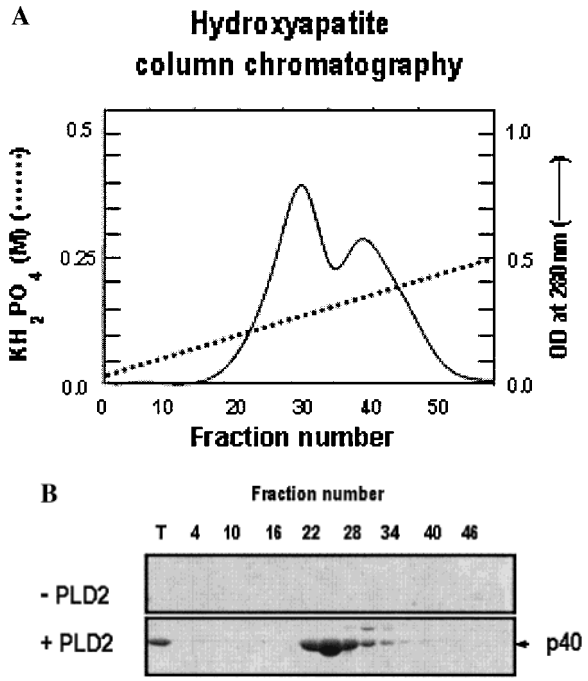
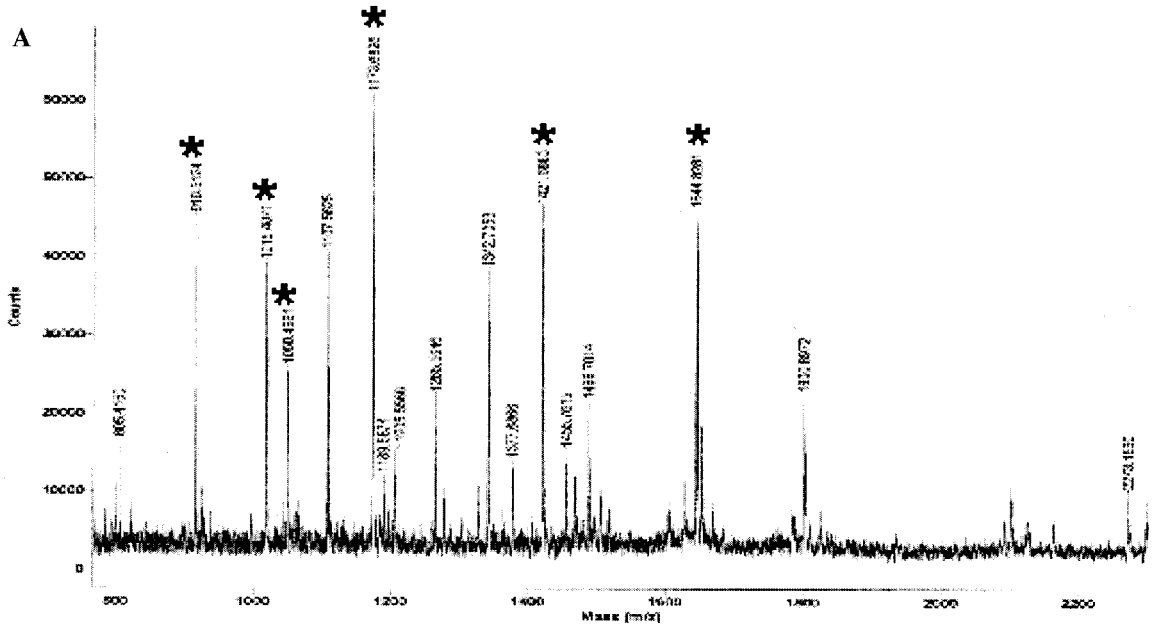


FIGURE 1: Isolation of PLD2-binding protein (p40) by blot overlay assay. Rat brain cytosol was subjected to sequential chromatography on Q-Sepharose, Phenyl Sepharose, Hi-Trap heparin, and hydroxyapatite column chromatography. Detailed procedures can be found under Experimental Procedures. (A) Protein profile of hydroxyaptite column chromatography. (B) The fractions from hydoroxyapatite column chromatography were subjected to blot overlay assay.

40 kDa PLD2-Interacting Protein Was Identified as Aldolase C. To identify the PLD2-interacting protein, p40 was excised from a polyacrylamide gel and digested with trypsin, and the cleaved peptide mixture was subjected to peptide-mass fingerprinting using matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF-MS). Figure 2A shows the MALDI mass spectrum of the digested peptides of p40. The masses obtained, marked as 1−6, were compared to proteins in the Swiss-Prot database using the MS−Fit peptide mass search program. As shown in Figure 2B, the peptides exhibited molecular masses that were almost identical to the calculated masses of the corresponding theoretically predicted tryptic peptides of aldolase C. This peptide search result was performed at an accuracy of 50 ppm, and the analyzed peptides covered 17% of the aldolase C sequence. Based on these results, we concluded that the 40 kDa protein interacting with PLD2 from the rat brain extract is aldolase C.

Aldolase A Interacts Specifically with PLD2 in COS-7 Cells. To further confirm the interaction between PLD2 and aldolase, GST−aldolase A was constructed. A similar pattern of interaction was observed with purified aldolase A (Sigma)



B PHSYPALSAE QKKELSDIALRIVAPGKGIL AADESVGSMA KRLSQIGVEN
TEENRRLYRQ VLFSADDRVK KCIGGVIFFH ETLYQKDDNG VPFVRTIQEK
GILVGIKVDKGVVPLAGTDGETTTQGLDGLLERCAQYKKD GADFAKWRCV
LKISDRTPSALAILENANVLARYASICQQNGIVPIVEPEILPDGDHDLKRQFV
EKVLAAVYKALSDHHVYLEGTLLKPNMVTPGHACPIKYSPEEIAMTALRRTV
PPAVPGVTFLSGGQSEEEASLNLNAINRCSLPRPWALTFSYGRALQASALS
AWRGQRDNAGAATEEFIKRAEMNGLAAQGKYEGSGDGGAAAQSLYVAN
AY

FIGURE 2: Identification of p40 as aldolase. (A) Peptide mixtures obtained after in-gel digestion of the excised band with trypsin were analyzed by MALDI-TOF-MS. The peptide masses labeled with an asterisk were matched with the calculated tryptic peptide masses of aldolase within 50 ppm. (B) Coverage map for aldolase. The matching peptide masses are underlined.

as with aldolase C in the blot overlay assay (data not shown). As shown in Figure 3A, GST−aldolase A interacted directly with PLD2 whereas GST did not interact with PLD2. To verify its potential in vivo interaction with aldolase A, human PLD2 was expressed in COS-7 cells, and the protein complex containing PLD2 was isolated using anti-PLD2 antibody as a probe. As shown in Figure 3B, endogenous aldolase was co-immunoprecipitated with PLD2 in COS-7 cells. Furthermore, COS-7 cells overexpressing PLD2 and Flag-tagged aldolase A were subjected to immunoprecipitation using anti-Flag antibody, and PLD2 was also shown to interact with aldolase A (Figure 3C). In summary, these results suggest that aldolase A probably forms a complex with PLD2 in COS-7 cells.

PLD2 Interacts Directly with Aldolase via Its PH Domain. To identify the region of PLD2 responsible for binding to aldolase A, we prepared GST-fusion protein of the PLD2 fragments and carried out the pull-down assay. The individual GST fragments of PLD2 are depicted in Figure 4A. As shown in Figure 4B, aldolase A interacted with an N-terminal region (amino acids, a.a., 1−314) of PLD2 but not with other regions. To further substantiate the region of PLD2 required for binding to aldolase A, GST-fusion proteins of the phox (PX) domain (a.a. 65−192) and the pleckstrin homology (PH) domain (a.a. 201−310) of PLD2 were separately generated. As shown in Figure 4C, aldolase A associated with the PH domain of PLD2 but not with the PX domain. These results led us to the conclusion that the PH domain of PLD2 may be essential for direct interaction with aldolase A.

Interaction between Aldolase A and the PH Domain of PLD2 Is Potentiated by Aldolase Metabolites. According to several previous reports, interaction between aldolase and its binding proteins has been known to be affected by aldolase metabolites (*26*−*28*). The interaction of aldolase with proteins such as F-actin, microtubules, and Glut 4 was disrupted by its metabolites. In this context, the effect of aldolase metabolites on the interaction between aldoalse A and the PH domain of PLD2 was examined. As shown in Figure 5A, binding of aldolase A to the PH domain of PLD2 was enhanced by increasing the concentration of the aldolase metabolites, fructose 1,6-bisphosphate (F-1,6-P) and glyceraldehyde 3-phosphate (G-3-P). In contrast, two other related sugars, dihydroxyacetone phosphate (DHAP) and fructose 1-phosphate (F-1-P), had no effect on the interaction between aldolase and the PH domain of PLD2. These results raise

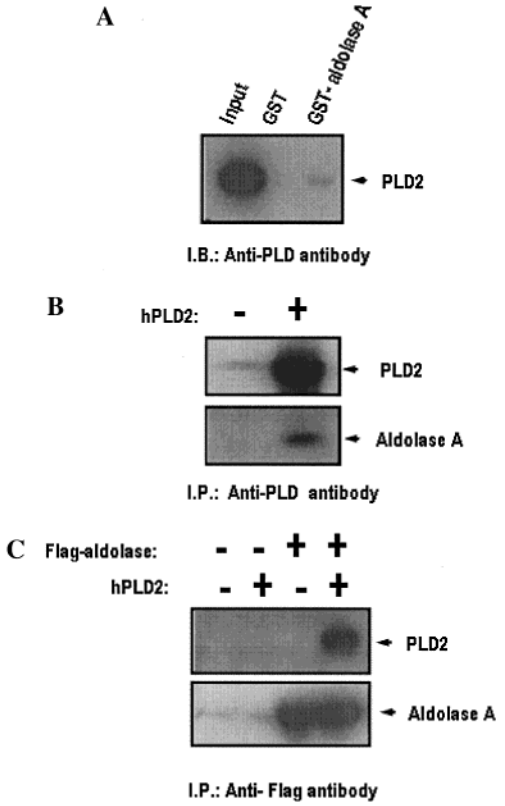


FIGURE 3: Association between PLD2 and aldolase. (A) Purified PLD2 was incubated with GST−aldolase. After a brief centrifugation and washing, the precipitates were analyzed by immunoblot analysis with anti-PLD2 antibody. GST was used as control. The data represent one of two independent experiments. (B) COS-7 cells were transfected with empty vector and pCDNA3.1 vector containing human PLD2. After a 48 h recovery period, the cells were subjected to immunoprecipitation with anti-PLD antibody. Precipitates were analyzed by immunoblot analysis with anti-PLD2 antibody (upper panel) or anti-aldolase antibody (lower panel). The data represent one of two independent experiments. (C) COS-7 cells were transfected with the combination of empty vector, pCDNA3.1 vector containing human PLD2, and pCMV vector containing aldolase. After a 48 h recovery period, the cells were subjected to immunoprecipitation with anti-Flag antibody. The precipitates were analyzed by immunoblot analysis with anti-PLD antibody (upper panel) or anti-Flag antibody (lower panel). The data represent one of two independent experiments.

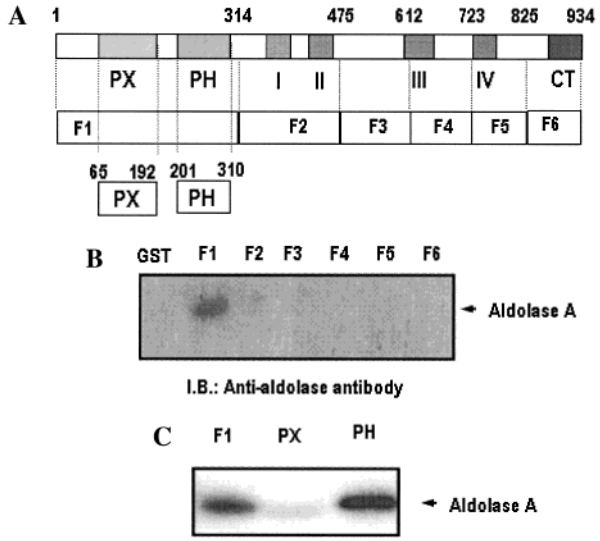


FIGURE 4: Direct interaction between PLD2 and aldolase through the PH domain of PLD2. (A) Primary structure and individual domains of PLD2. GST-fusion proteins containing different regions of PLD2 were expressed in bacteria and purified by glutathione Sepharose beads. The individual domains of PLD2 fragments were followed by F1 (a.a. 1−314), F2 (a.a. 315−475), F3 (a.a. 476−612), F4 (a.a. 613−723), F5 (a.a. 724−825), F6 (a.a. 826−934), the PX domain (a.a. 65−192), and the PH domain (a.a. 201−310). Boxes indicate regions of highly conserved sequences in PLD isozymes. PX, phox domain; PH, pleckstrin homology domain; CR I, II, III, IV, conserved region I, II, III, IV; CT, carboxyl terminus. (B) Aldolase (500 nM) was incubated with equal amounts (1 μg) of GST or GST-fusion proteins (GST-PLD2 fragments, F1−F6) as described under Experimental Procedures. After a brief centrifugation and washing, the precipitates were analyzed by immunoblot analysis with anti-aldolase antibody. GST was used as control. The data represent one of two independent experiments. (C) Aldolase (500 nM) was incubated with equal amounts (1 μg) of GST-PX domain or the GST-PH domain of PLD2. After a brief centrifugation and washing, the precipitates were analyzed by immunoblot analysis with anti-aldolase antibody. The data represent one of three independent experiments.

the possibility that the interaction that exists between PLD2 and aldolase A can be modulated by responding to the changes of the concentration of aldolase metabolites, F-1,6-P and G-3-P.

Aldolase A Specifically Interacts with the PH Domain of PLD2. Until now, a few of the protein binding partners have been identified for the PH domain, although its interaction with phosphoinositides has been well-documented. Considering the specificity of the PH domain of PLD2 in the interaction with aldolase, possible interaction with other PH domains, found in the pleckstrin N- or C-terminus, phospholipase C δ1 (PLC δ1), diacylglycerol kinase (DGK), and Akt 1, has been analyzed. Aldolase A binds specifically to the PH domain of PLD2 but not to the other PH domains (Figure 6). Even at the condition optimized for the interaction between the PH domain of PLD2 and aldolase, the presence of aldolase metabolites, any detectable binding to aldolase was not observed with other PH domains. The observed specificity of the PH domain in the interaction of PLD2 with aldolase can be explained by a very low degree of overall sequence similarity among the PH domains.

Aldolase A Inhibits PLD2 Activity and Its Inhibitory Activity on PLD2 Is Further Potentiated by Its Metabolites. The effect of aldolase A on the activity of PLD2 was examined to understand the biological meaning of the interaction between two molecules. As shown in Figure 6, aldolase A inhibits PLD2 activity in vitro in a concentration-dependent manner, with an IC_{50} of approximately 1 μM in the absence of its metabolites. Reminiscent of the increased interaction of aldolase A with the PH domain of PLD2 by the presence of its metabolites, we further examined the effect of the metabolites on aldolase A-mediated PLD2 inhibition. The introduction of aldolase metabolites resulted in enhancement of the PLD2-inhibitory ability of aldolase, approximately 200 nM aldolase A required for half-maximal inhibition. The metabolites, in themselves, had no effect on PLD2 activity under the same assay conditions, which suggests that the significant inhibition of PLD2 by metabolites may result from the effect of metabolites on aldolase A, but not from that on PLD2. From these results, it is demonstrated that aldolase A inhibits PLD2 activity in vitro through the specific interaction with the PH domain of PLD2 which is potentiated by the increasing concentration of aldolase metabolites.

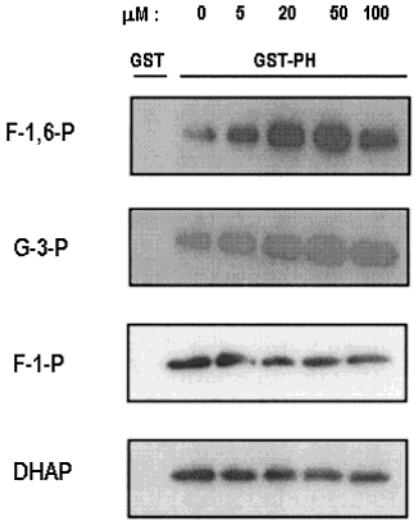


FIGURE 5: Effects of aldolase metabolites on the binding between the PH domain of PLD2 and aldolase. Aldolase (100 nM) was incubated with the GST-PH domain of PLD2 in the presence of the indicated amounts of fructose 1,6-bisphosphate (F-1,6-P), glyceraldehyde 3-phosphate (G-3-P), fructose 1-phosphate (F-1-P), or dihydroxyacetone phosphate (DHAP). After a brief centrifugation and washing, the precipitates were subjected to immunoblot analysis with anti-aldolase antibody. The data represent one of two independent experiments.

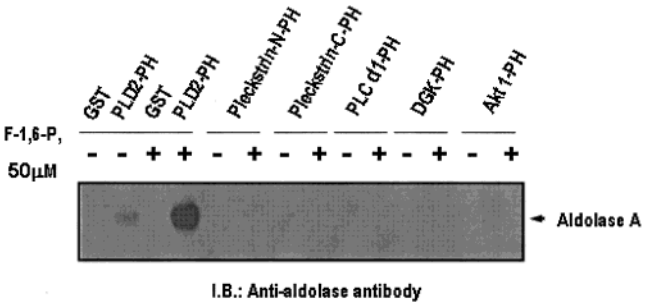


FIGURE 6: Aldolase binding specificity of the PH domain of PLD2 and other PH domains. Aldolase (100 nM) was incubated with equal amounts (1 μg) of GST or the GST-PH domain of PLD2, pleckstrin N- or C-terminus, phospholipase δ1 (PLC δ1), diacylglycerol kinase (DGK), or Akt 1 in the absence (−) or the presence (+) of fructose 1,6-bisphosphate (50 μM). After a brief centrifugation and washing, the precipitates were subjected to immunoblot analysis with anti-aldolase antibody. The data represent one of two independent experiments.

DISCUSSION

To understand the molecular details of the PLD-mediated signaling pathway by systematically organizing the network of protein−protein interactions, dozens of research groups, including ours, have approached the subject using several methods, such as yeast two-hybrid screening, immunoprecipitation with anti-PLD antibody, affinity purification using immobilized chimeric fusion protein with a tag, and blot overlay assay. In this study, PLD2-interacting proteins were isolated using the blot overlay assay with purified PLD2. As a result, a protein with a molecular mass of 40 kDa was identified as aldolase C by peptide mass analysis using MALDI-TOF-MS. Furthermore, the metabolite-induced increase of the interaction of aldolase with the PH domain of PLD2 resulted in the enhancement of the inhibitory potential of aldolase on PLD2 in vitro. To the best of our knowledge, this report is the first to suggest the possibility that glycolytic

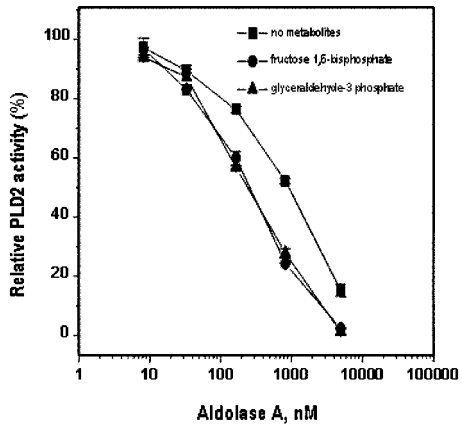


FIGURE 7: Inhibition of PLD2 activity by aldolase and its potentiation by aldolase metabolites. PLD2 activity was measured in the presence of varying concentrations of aldolase (■). Fructose 1,6-bisphosphate (●) or glyceraldehyde 3-phosphate (▲) was added to a final concentration of 50 μM. The assay was performed as described under Experimental Procedures. The data represent means ± SE of two independent experiments performed in duplicate.

intermediates can transduce the signal required for regulating the production of lipid second messengers by modulating specifically the interaction between aldolase and PLD2.

During the course of the research performed to identify the PLD-interacting proteins, there have been debates on the issue concerning that the association takes place through direct interaction. In this report, there are several experimental evidences for supporting a direct interaction between aldolase A and PLD2. First, GST−aldolase A fusion protein interacts with purified PLD2 in the pull-down assay (Figure 3A). Second, the inhibitory activity of aldolase A on PLD2 inhibition was unaffected by the presence or absence of PIP_2 (data not shown), which suggests that the PLD2-inhibitory effect of aldolase A is not due to PIP_2 sequestration or masking. Finally, the PH domain (a.a. 201−310) of PLD2 is essential for the interaction with purified aldolase A (Figure 3C,D).

Aldolase specifically binds to the PH domain of PLD2, but not to PH domains found in other signaling proteins, such as the pleckstrin N- or C-terminus, phospholipase C δ1 (PLC δ1), diacylglycerol kinase (DGK), and Akt 1 (Figure 6). PH domains, which were first identified in pleckstrin, are small protein modules of around 100−120 residues found in a wide range of proteins implicated in cellular signaling, cytoskeleton rearrangement, and other physiological processes. While several different proteins have been reported to be associated with the PH domain through the intensive study performed to determine the protein−protein interaction mediated by the PH domain, the only clearly defined physiological function is to direct membrane targeting of the host protein by binding to phosphoinositides. β/γ subunits of the heterotrimeric G protein have been proposed to interact with PH domains found in a dozen signaling proteins including β-adrenergic receptor kinase and Bruton tyrosine kinase (*29*, *30*). RACK1 (Receptor for Activated C-Kinase) binds to selective PH domains, and the interaction between RACK1 and the PH domain was found to be unaffected by either IP_3 or PIP_2 (*31*). Information on the PH domain-specific protein−protein interaction remains very limited, and its physiological roles remain to be discovered. When one considers that the PH domains show

only a very low degree of overall sequence similarity compared with other domains reported so far, the unique structural motif residue in the PH domain of PLD2 might be required for the specific association between PLD2 and aldolase. Intensive mutagenic analysis is needed to narrow-down the amino acids in the PH domain of PLD2 that participate in the specific interaction with aldolase.

In this report, we first identified aldolase, an enzyme in the glycolytic pathway other than the signaling process, as a target protein of the PH domain of PLD2. Aldolase is a well-known glycolytic enzyme that catalyzes the reversible cleavage of fructose 1,6-bisphosphate (F-1,6-P) to glyceraldehyde 3-phosphate (G-3-P) and dihydroxyacetone phosphate (DHAP) (*32*). Several research groups have demonstrated that aldolase affects the cellular signaling process, including the acidification of intracellular compartments, which is carried out by vacuolar H^+-ATPase (V-ATPase) (*33*) and insulin-dependent glucose transport mediated by GLUT4 (*26*), through specific protein−protein interaction. The carboxyl terminal of GLUT4, but not GLUT1, has been reported to interact specifically with aldolase, and their partial co-localization was demonstrated in 3T3L1 adipocytes. Aldolase was suggested to play a role as a molecular scaffold linking GLUT4 with F-actin by mediating F-actin−aldolase−GLUT4 ternary complex formation, which can be completely abolished by the addition of increasing amounts of aldolase substrates in vitro. Furthermore, the introduction of fructose 1,6-bisphosphate or the glycolytic pathway inhibitor 2-deoxyglucose into permeabilized 3T3L1 adipocytes inhibited insulin-stimulated GLUT4 translocation as well as the interaction between aldolase and F-actin (*26*). According to a recent report, microinjection of bacterial PLD into cultured adipocytes resulted in marked stimulation of the effect of insulin on GLUT4 translocation to the cell surface membrane (*9*). Moreover, PLD1 partially co-localized with GLUT4 in both 3T3L1 adipocytes and Chinese hamster ovary cells, and the co-localization was disrupted by the introduction of PLD1 mutant lacking the PH domain, which suggests a pivotal role of the PH domain of PLD1 in the association of PLD1 with GLUT4 translocation. Although the functional implication of PLD activity in insulin-mediated GLUT4 translocation has been demonstrated in this report, the question raised concerning the role of the PH domain-mediated association of PLD1 with GLUT4 in GLUT4 translocation has not been addressed.

Considering the results from two separate previous reports, which suggest the functional links of GLUT4 with aldolase and with PLD, a missing link between PLD and aldolase was proved in this report. A possible regulatory link between PLD and aldolase was first suggested by our finding that aldolase is one of the PLD2-interacting proteins and that PLD activity is regulated by aldolase through its specific interaction with the PH domain of PLD2 responding to the availability of its metabolites. Taken together, a ternary signaling complex composed of PLD, aldolase, and GLUT4, in which the biological activities and location of its constituents are regulated by the dynamic changes of the association among the molecules, might be proposed. In this working model of the ternary complex, insulin-dependent glucose uptake can be adjusted depending on the energy status of the cell, the availability of glycolytic intermediates; the increase of PLD activity, which takes place by insulin treatment, is attenuated by the metabolite-induced increase of the binding of aldolase to PLD, and thereby leads to the inhibition of GLUT4 translocation and glucose uptake, when enough of the glycolytic intermediates is available in cells. To characterize the physiological function of the interaction between PLD2 and aldolase in detail, the effect evoked by treatment of fructose 1,6-bisphosphate or 2-deoxyglucose into intact cells must be analyzed with respect to PLD activity and to insulin-dependent GLUT4 translocation and glucose uptake. Protein−protein interactions reside in ternary complex formation, and their insulin-dependent changes should also be monitored in intact cells exposed in a different energy status.

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