Identification of a Cellular Protein That Functionally Interacts with the C2 Domain of Cytosolic Phospholipase A2α

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Cytosolic phospholipase A2 (cPLA2)α plays critical roles in lipid mediator synthesis. We performed far-Western analysis and identified a 60-kDa protein (P60) that interacted with cPLA2α in a Ca2+-dependent manner. Peptide microsequencing revealed that purified P60 was identical to vimentin, a major component of the intermediate filament. The interaction occurred between the C2 domain of cPLA2α and the head domain of vimentin. Immunofluorescence microscopic analysis demonstrated that cPLA2α and vimentin colocalized around the perinuclear area in cPLA2α-overexpressing human embryonic kidney 293 cells following A23187 stimulation. Forcible expression of vimentin in vimentin-deficient SW13 cells augmented A23187-induced arachidonate release. Moreover, overexpression of the vimentin head domain in rat fibroblastic 3T3 cells exerted a dominant inhibitory effect on arachidonate metabolism, significantly reducing A23187-induced arachidonate release. These results suggest that vimentin is an adaptor for cPLA2α to function properly during the eicosanoid-biosynthetic process.

Phospholipase A2 (PLA2)1 hydrolyzes the ester bonds of fatty acids present at the sn-2 positions of phospholipids. PLA2 plays crucial roles in diverse cellular responses, including phospholipid digestion and metabolism, host defense, signal transduction, and probably apoptosis. PLA2α provides precursors for the biosynthesis of eicosanoids, such as prostaglandins (PGs) and leukotrienes, when the hydrolyzed fatty acid is arachidonic acid (AA); platelet-activating factor when the sn-1 position of phosphatidylcholine contains an alkyl ether linkage; and some bioactive lysophospholipids, such as lysophosphatidic acid. As oversynthesis of these lipid mediators causes inflammation and tissue disorders, it is important to elucidate the mechanisms that regulate the functions of PLA2. Mammalian tissues and cells generally contain more than one PLA2, each of which is regulated independently and plays distinct roles. There are three large families of mammalian PLA2s, cytosolic PLA2 (cPLA2), secretory PLA2 (sPLA2), and Ca2+-independent PLA2, and the roles of type IV cPLA2α and secretory types IIA and V sPLA2 in lipid mediator synthesis have been studied extensively (1–3).

An 85-kDa type IV cPLA2α appears to be one of the most important PLA2 isoforms involved in lipid mediator synthesis. We found that vimentin, a major component of the intermediate filament, also play roles in cPLA2α activation. This enzyme preferentially hydrolyses phospholipids bearing AA (4, 5). cPLA2α is expressed in most mammalian cells, and its activation is regulated by several postreceptor signal transduction events, such as Ca2+ mobilization (6, 7), phosphorylation (8, 9), and gene induction (10, 11). After stimuli that are accompanied by an increase in the cytoplasmic Ca2+ concentration, cPLA2α translocates rapidly and often transiently to the perinuclear and endoplasmic reticular membranes (12, 13), is phosphorylated by mitogen-activated protein kinases (8, 9), and releases AA for immediate conversion to PGs and leukotrienes by the constitutive cyclooxygenase (COX)-1 and lipooxygenase (5-LO), respectively (14–17). Furthermore, cPLA2α has been implicated in the inducible COX-2-dependent delayed PG generation, which lasts for hours, initiated by proinflammatory stimuli, such as interleukin-1, tumor necrosis factor α, and lipopolysaccharide (18–21). cPLA2α has several functionary distinct regions: an amino-terminal Ca2+-dependent lipid binding domain (amino acids 18–138) called the C2 domain (22, 23), a carboxyl-terminal region (amino acids 179–749) containing the catalytic domain, a putative pleckstrin homology domain (amino acids 263–354) similar to phospholipase C-δ1 (24), and two critical serine residues (Ser205 and Ser207), which undergo activation-directed phosphorylation (25). The C2 domain is responsible for translocation of cPLA2α from the cytosol to the membrane compartment and exhibits significant homology with the C2 domains of several proteins, such as protein kinase C, GTPase-activating protein, synaptopagmin, and phospholipase C, all of which bind to phospholipid membranes in a Ca2+-dependent manner (22, 23). The carboxy-terminal region mediates the Ca2+-independent enzymatic catalysis, which involves Ser207 at the active site (26). Interestingly, a number of enzymes involved in AA metabolism, such as COX-1 and -2, 5-LO, and 5-LO-activating protein, are also localized in the nuclear envelope and endoplasmic reticulum (27–29).

Although the C2 domain appears to be responsible for Ca2+-dependent localization of cPLA2α to its membrane substrates, whether additional interactions, such as C2 domain binding to an adaptor protein, also play roles in cPLA2α targeting to particular membrane compartments remains to be elucidated. In this study, we carried out far-Western screening in an attempt to determine how cPLA2α translocates specifically to the perinuclear particles and identified a protein that interacted with cPLA2α. We found that vimentin, a major component...
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protein of intermediate filaments, interacted with the amino-terminal region of cPLA2a in a Ca2+-dependent manner. Further experiments demonstrated that cPLA2α and vimentin colocalized in Ca2+-ionophore-stimulated, but not unstimulated, human embryonic kidney 293 cells stably transfected with cPLA2α. Introduction of vimentin into a vimentin-deficient SW13 cell line restored cPLA2α-dependent AA release in response to a Ca2+-ionophore, whereas introduction of the vimentin head domain, to which cPLA2α bound via its C2 domain, into rat fibroblastic 3Y1 cells reduced it. These results suggest that vimentin is involved in the regulation of cPLA2α in the AA metabolic pathway.

Experimental Procedures

Materials—Mouse cPLA2α cDNA was provided by Dr. Tsujimoto (RIKEN Institute); the FGE2 enzyme immunoassay kit was purchased from Cayman Chemical; and mouse monoclonal anti-glutathione S-transferase (GST), anti-human cPLA2α, and anti-human vimentin antibodies were purchased from Sigma, Novagen, and CLONTECH, respectively, and A23187 was purchased from Calbiochem.

Cell Culture—Adherent macrophages were prepared from the peritoneal cells of male Harlan Sprague-Dawley rats (Nippon Bio-Supply Center) 4 days after the injection of 5% (w/v) soluble starch and 5% (w/v) Bacto-peptone in saline (5 ml/100 g of body weight) as described previously (30) and cultured in RPMI 1640 medium (Nissui) supplemented with 10% (v/v) fetal calf serum. All the above cells were cultured in a 5% CO2 humidified atmosphere at 37°C. Sf9 cells (Institute of Development, Aging, and Cancer, Sendai) were grown in DMEM supplemented with 10% fetal calf serum. All the above cells were cultured in a 5% CO2 humidified atmosphere at 37°C. Sf9 cells (Institute of Development, Aging, and Cancer, Sendai) were grown in DMEM supplemented with 10% fetal calf serum. Adherent macrophages were prepared from the peritoneal cells of male Harlan Sprague-Dawley rats (Nippon Bio-Supply Center) 4 days after the injection of 5% (w/v) soluble starch and 5% (w/v) Bacto-peptone in saline (5 ml/100 g of body weight) as described previously (30) and cultured in RPMI 1640 medium (Nissui) supplemented with 10% (v/v) fetal calf serum. Rat fibroblastic 3Y1 cells (donated by Dr. Uehara, National Institute of Health, Tokyo, Japan), human adenocortical carcinoma SW13 cells (donated by Dr. D. M. Marcus, Baylor College of Medicine) and human glioblastoma U251 cells (obtained from RIKEN Cell Bank) were grown in Dulbecco’s modified Eagle’s medium (Nissui) supplemented with 10% fetal calf serum. Mouse osteoblastic MC3T3-E1 cells (obtained from RIKEN Cell Bank) and human embryonic kidney 293 cells (obtained from the Health Science Research Resources Bank) were grown in a-modified Eagle’s medium (ICN Biomedicals) and RPMI 1640 medium, respectively, both of which were supplemented with 10% fetal calf serum. All the above cells were cultured in a 5% CO2 humidified atmosphere at 37°C. Sf9 cells (In-vitrogen) were cultured in Grace’s insect medium (Invitrogen) supplemented with 10% fetal calf serum.

Preparation of Recombinant Epitope-tagged Native and Truncated cPLA2α Proteins—Mouse cPLA2α cDNA was subcloned into the EcoRI restriction site of the bacterial expression vector pGEX2T (Amersham Pharmacia Biotech). Truncated mutants of cPLA2α protein were prepared by the polymerase chain reaction (PCR) with ex Taq polymerase (Takara Shuzo) and mouse cPLA2α cDNA as the template. The following primer pairs were designed: cPLA2α (1–138), 5′-GCC GAC ATC TCT TCT ATC GAT CCT TAT C, and MC-138, 5′-CTC GCC GAC ATC TCT ATC GAT CCT TAT C, and MC-138, 5′-CTC GCC GAC ATC TCT ATC GAT CCT TAT C. The resulting construct was subcloned into the prokaryotic expression vector pGEX4T3 (Amersham Pharmacia Biotech).

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This plasmid was transformed into E. coli strain BL21(DE3) (Stratagene), a lon mutant strain containing T7 polymerase under the control of the lacUV5 promoter, and 1 mm isopropyl β-D-thiogalactopyranoside was added to induce expression of T7-tagged cPLA2α at 37°C. A crude fraction of the expressed T7-tagged cPLA2α was obtained by solubilization in inclusion bodies with 8 M urea (in 100 mM Tris pH 7.4) for 20 min, and the solubilized fraction was used for far-Western analysis.

Site-directed Mutagenesis of cPLA2α cDNA—Site-directed mutations of cPLA2α cDNA were introduced by transfecting mutant PCR with ex Taq polymerase using truncated mouse cPLA2α (1–138) inserted in the pCR3.1 vector as the template. In order to obtain site-directed mutants, the PCR products obtained from the first PCR using the following mutated primer pair: D43N (5′-GG ACA CTC CAA ATC TTT AGG, in which Asp43 was replaced by Asn at the underlined site) and D93N (5′-GTT GGC ATT CAT CAG TGT G, in which Asp93 was replaced by Asn at the underlined site) were mixed, denatured at 95°C for 5 min, and then annealed with gradual cooling to 37°C. The second PCR was performed using the amino-terminal sense and MC-138 antisense primer pair and the annealed sample as the template. The product was subcloned into the PCRI.1 vector and the mutation of the construct was verified by DNA sequencing. A double mutant was obtained by cloning the 0.2-kilobase pair BamHI fragment of PCRI.1- cPLA2α (1–138) D93N, which contains a single mutation, into the 5.2-kilobase pair BamHI fragment of PCRI.1-cPLA2α (1–138) D43N. The truncated construct was cloned into pGEXKT3 and expressed as a GST fusion protein, as described above.

Preparation of Recombinant Vimentin Proteins—Vimentin cDNA was amplified by performing the reverse transcription-PCR using poly(A)+ RNA purified from rat fibroblastic 3Y1 cells, and the primer pair, synthesized according to the rat vimentin cDNA sequence, composed of the following sequences: 5′-GG AATTC CATGTCCACCAGG- TCCCGT and 5′-GG AATTC TCAAGGTCATCGTGGTGC. The red and tail domains of vimentin protein were prepared by the PCR using full-length vimentin cDNA as the template. The following primer pairs were designed: vimentin (95–406), 5′-AG GAAATTCGATGAATCCACGACC- GCC and 5′-CC AAAGCTTCTCCCGACGGTTT; and vimentin (407–466), 5′-GG GAAATTCGATGAATCCACGACC- GCC and 5′-GG GAAATTCGATGAATCCACGACC- GCC. Initially, the amplified fragments were cloned into the pCR1.1 vector and sequenced as described above, and the inserts were subcloned into the prokaryotic expression vector pET21a (Novagen). The head domain of vimentin protein was released from PCRI.1-vimentin by restriction enzyme digestion (EcORI and XhoI) and subcloned into the pET21a vector, which expressed protein as a fusion protein containing a stretch of 6 consecutive histidine residues at the amino-terminal end of the carboxy-terminal domain. The plasmid was transformed into E. coli BL21(DE3), and 1 mm isopropyl β-D-thiogalactopyranoside was added to induce expression of the His-tagged vimentin protein, which was purified using Ni-NTA agarose (Qiagen) according to the manufacturer’s instructions and used for the subsequent experiments.

SDS-Polyacrylamide Gel Electrophoresis (PAGE)/Immunoblotting—Cultured cells were washed once with 10 mM phosphate buffer (pH 7.4) containing 1 mM EDTA and 1% (w/v) Triton X-100, lysed by sonication. The lysates were subjected to SDS-PAGE under reducing conditions and electrotitransferred to nitrocellulose membranes (Schleicher & Schuell) using a semidyry blotter (milliBLOT-SIDE system; Millipore), according to the manufacturer’s instructions. The membranes were washed once with H2O, blocked with 5% (w/v) skim milk in PBS containing 0.05% (w/v) Tween 20 (PBS-T) for 1 h at room temperature or overnight at 4°C, and washed with PBS-T. Then, the mouse monoclonal anti-vimentin, anti-GFAP, and anti-EGFP antibodies (diluted 1:5,000, 1:2000, and 1:1000, respectively, with PBS-T containing 1% (w/v) skim milk) were added, and the membranes were incubated for 2 h at room temperature, washed three times with PBS-T, and treated with horseradish peroxidase-conjugated goat anti-mouse IgG (Zymed Laboratories Inc.; diluted 1:3,000) in PBS-T containing 1% (w/v) skim milk for 1 h at room temperature. Finally, after six washes with PBS-T, the protein bands were visualized using the ECL Western blot analysis system (Amersham Pharmacia Biotech).

Far-Western Analysis—Samples were subjected to SDS-PAGE and electrotitransferred to nitrocellulose membranes, which were blocked with 5% (w/v) skim milk in PBS-T for 1 h at room temperature or overnight at 4°C. Then, the nitrocellulose membranes were incubated with 10% (w/v) skim milk for 1 h and washed with PBS-T. After washing with PBS-T containing 1% (w/v) skim milk for 1 h, the membranes were incubated with 1 μg/ml purified GST-cPLA2α or GST (control). The bound proteins were immunoblotted with the anti-GST monoclonal antibody and then incubated with horseradish peroxidase-conjugated anti-mouse IgG, as described above. When T7-tagged cPLA2α was used instead of GST-cPLA2α, the protein band was visualized using the monoclonal anti-T7
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**Antibody** (Novagen; diluted 1:2000).

**Baculovirus Expression of Recombinant cPLA \(_2\alpha\)-Mouse cPLA \(_2\alpha\)** cDNA was subcloned into the baculovirus transfer vector pVL1393 (Pharmlingen), and S9 cells were infected with the resulting baculovirus, as described elsewhere (31, 32). After culture at 27 °C for 4 days, the cells containing cPLA \(_2\alpha\) were harvested, lysed by sonication, and centrifuged at 100,000 \(\times\) g for 4 h. The supernatant was introduced to a DEAE-Sephacel column (10 \(\times\) 50 mm) (Amersham Pharmacia Biotech) preequilibrated with Tris-buffered saline (pH 8.0) and centrifuged at 10,000 \(\times\) g for 4 h. The supernatant was then loaded onto a Glutathione-Sepharose 4B column and eluted with glutathione at a linear gradient of 50–0 mM NaCl at a flow rate of 25 m\(\cdot\)h. The fractions containing cPLA \(_2\alpha\) were eluted as a major protein peak and pooled for subsequent experiments.

**Partial Purification of P60**—3Y1 cells (3 \(\times\) 10\(^8\)) were harvested in PBS containing trypsin/EDTA and washed in PBS, and the pelleted cells were suspended in 10 ml of ice-cold lysis buffer (10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 1.5 mM MgCl\(_2\), 1% (v/v) Nonidet P-40, and 10 \(\mu\)g/ml leupeptin). The first centrifugation at 12,000 \(\times\) g for 5 min at 4 °C, resuspended in 1 ml of 50 mM Tris-HCl (pH 7.4) containing 25 mM KCl and 5 mM MgCl\(_2\), and sonicated briefly, and solid tripotassium citrate was added to produce a final concentration of 10% (w/v). Then, the sample was centrifuged at 35,000 \(\times\) g for 45 min at 4 °C to obtain a chromatin-rich fraction (supernatant) and a nuclear matrix-rich fraction (pellet), both of which were used for far-Western analysis, as described above.

**Sequence of P60**—The P60 band on the first SDS-PAGE gel was visualized using Coomasie Brilliant Blue, cut out from the gel, and then partially digested in the second gel with 10 \(\mu\)g of Achromobacter lysyloendopeptidase (Wako). The digested peptides were electrophoresed on a polyvinylidene difluoride membrane (Millipore), and the two major peptide fragments obtained were subjected to amino-terminal amino acid sequencing using an Applied Biosystems 473A Protein Sequencer. Degenerate PCR primers based on the amino acid sequences were designed, and the PCR was performed (30 cycles each comprising 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s) using 3Y1 cDNA as the template. The sequence of a major DNA fragment amplified was sequenced using the BLAST program.

**Coprecipitation Assays—GST-cPLA \(_2\alpha\)** (1–138)-bound glutathione-Sepharose CL-4B was incubated with purified recombinant rat vimentin (1 μg) in 2 ml of 50 mM Tris-HCl (pH 7.5) containing 1 mM MgCl\(_2\) and 1 mM dithiothreitol, and the bound proteins were eluted by incubation with 20 mM glutathione for 30 min at 4 °C. The eluted fractions were subjected to SDS-PAGE, transblotted to a nitrocellulose membrane, and immunoblotted with the anti-vimentin monoclonal antibody, as described above.

**Immunofluorescence Microscopy**—The subcellular distribution patterns of cPLA \(_2\alpha\) and vimentin in 293 cells stably transfected with mouse cPLA \(_2\alpha\), which were established as described previously (33), were analyzed using a double indirect immunofluorescence staining technique, using most of the C2 domain (cPLA \(_2\alpha\) amino acid sequence) as a probe. In this assay, the filters onto which the cellular proteins were transferred were incubated with either GST or GST-cPLA \(_2\alpha\) and then blotted with an anti-GST antibody. No obvious bands were detected after incubation with GST alone, whereas a protein with a molecular mass of approximately 60 kDa was detected, after incubation with GST-cPLA \(_2\alpha\), in the lysates of rat peritoneal macrophages and mouse osteoblastic MC3T3-E1 cells (Fig. 1A). This protein, tentatively designated P60, was also detected in the lysates of MC3T3-E1 cells and rat fibroblastic 3Y1 cells (Fig. 1B) as well as macrophages (data not shown) when T7-tagged, instead of GST-tagged, cPLA \(_2\alpha\) was used as a probe and visualized using an anti-T7 antibody. Therefore, P60 appears to be a cPLA \(_2\alpha\)-binding protein.

**Results**

Detection of a cPLA \(_2\alpha\)-binding Protein—In an attempt to search for a protein that interacts with cPLA \(_2\alpha\), lysates of several cells that generated PGE\(_2\) in a cPLA \(_2\alpha\)-dependent manner (19, 34, 35) were subjected to far-Western analysis using epitope-tagged cPLA \(_2\alpha\) protein as a probe. In this assay, the filters onto which the cellular proteins were transferred were incubated with either GST or GST-cPLA \(_2\alpha\) and then blotted with an anti-GST antibody. No obvious bands were detected after incubation with GST alone, whereas a protein with a molecular mass of approximately 60 kDa was detected, after incubation with GST-cPLA \(_2\alpha\), in the lysates of rat peritoneal macrophages and mouse osteoblastic MC3T3-E1 cells (Fig. 1A). This protein, tentatively designated P60, was also detected in the lysates of MC3T3-E1 cells and rat fibroblastic 3Y1 cells (Fig. 1B) as well as macrophages (data not shown) when T7-tagged, instead of GST-tagged, cPLA \(_2\alpha\) was used as a probe and visualized using an anti-T7 antibody. Therefore, P60 appears to be a cPLA \(_2\alpha\)-binding protein.

To determine whether the C2 domain (amino acids 18–138) of cPLA \(_2\alpha\) was involved in the binding of cPLA \(_2\alpha\) to P60, we engineered a truncated cPLA \(_2\alpha\) cDNA construct, which was bacterially expressed as a GST fusion protein (GST-cPLA \(_2\alpha\) (1–138)). Far-Western analysis using GST-cPLA \(_2\alpha\) (1–138) as a probe revealed that the C2 domain of cPLA \(_2\alpha\) bound more effectively than full-length cPLA \(_2\alpha\) (Fig. 1C). Further carboxy-terminally truncated mutant cPLA \(_2\alpha\) (1–81) still retained the ability to interact with P60, whereas neither the mutant lacking most of the C2 domain (cPLA \(_2\alpha\) (1–81)) nor that with a truncated amino-terminal (cPLA \(_2\alpha\) (36–81)) displayed significant binding (Fig. 1D). Therefore, we used GST-cPLA \(_2\alpha\) (1–138) as a probe in subsequent experiments.

To verify that GST-cPLA \(_2\alpha\) (1–138) bound specifically to P60, we carried out far-Western analysis to determine whether recombinant cPLA \(_2\alpha\) prepared from a baculovirus-insect cell system competed with GST-cPLA \(_2\alpha\) (1–138) for binding to P60. Excess recombinant cPLA \(_2\alpha\) added to the reaction mixture inhibited markedly and competitively binding of GST-cPLA \(_2\alpha\) (1–138) to P60 (Fig. 1E), which suggests strongly that the interaction between cPLA \(_2\alpha\) and P60 was specific and saturable.

In order to examine the subcellular distribution of P60, 3Y1 cells were lysed with the nonionic detergent Nonidet P-40 and centrifuged at 12,000 \(\times\) g, and the resulting supernatant and precipitate were subjected to far-Western analysis. As shown in Fig. 1F, P60 was detected exclusively in the precipitate fraction, which contained chromatin and nuclear matrix.
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FIG. 1. Detection of a cPLA$_2$,$\alpha$-binding protein (P60) by far-Western analysis. The lysates (10-$\mu$g protein equivalents) of several cells were subjected to far-Western analysis using GST-tagged (A) and T7 peptide-tagged (B) cPLA$_2$. C, 3Y1 cell lysate was subjected to far-Western analysis using equivalent concentrations of GST-tagged native and truncated cPLA$_2$.$\alpha$. E, the effect of excess recombinant cPLA$_2$.$\alpha$ on the interaction between GST-cPLA$_2$.$\alpha$ and P60 in 3Y1 cells. The nitrocellulose membrane was incubated with GST-cPLA$_2$.$\alpha$ (1–138) as a probe in the presence (+) or absence (−) of more than 10 times excess recombinant cPLA$_2$.$\alpha$. F, the subcellular distribution of P60 in 3Y1 cells was assessed using GST-cPLA$_2$.$\alpha$ (1–138). Each fraction was prepared as described under “Experimental Procedures.” Lanes 1–4, Nonidet P-40-insoluble, Nonidet P-40-insoluble, chromatin-rich, and nuclear matrix-rich fractions, respectively.

this Nonidet P-40-insoluble fraction was separated into chromatin- and nuclear matrix-rich fractions, P60 was detected only in the latter (Fig. 1F).

Ca$^{2+}$ Requirement for cPLA$_2$.$\alpha$ Binding to P60—As the C2 domain is a Ca$^{2+}$-binding motif, we carried out far-Western analysis to examine the effects of Ca$^{2+}$-chelators on the binding of cPLA$_2$.$\alpha$ to P60. Virtually no P60 was detected in the presence of 5 mM EDTA (Fig. 2A) or EGTA (data not shown), indicating that this binding reaction may require Ca$^{2+}$, a significant amount of which was present in the skim milk used for membrane blocking.

C2 domains generally form two distinct topological folds, as exemplified by the crystal structures of these domains of synaptotagmin I (type I) and phosphoinositide-specific phospholipase C-61 (type II) (22, 23). cPLA$_2$.$\alpha$ belongs to the latter group, and the amino acid residues essential for its Ca$^{2+}$ binding are Asp$^{40}$, Thr$^{41}$, Asp$^{43}$, Asn$^{65}$, Asp$^{93}$, Ala$^{94}$, and Asn$^{95}$ (36). Therefore, we replaced two of these residues with other amino acids, D43N and D93N, as the resulting mutation has been shown recently to reduce the Ca$^{2+}$ sensitivity of cPLA$_2$.$\alpha$ markedly (37). Under the conditions that resulted in binding of native cPLA$_2$.$\alpha$ (1–138) to P60, this mutant cPLA$_2$.$\alpha$ (1–138) did not bind to P60 (Fig. 2B). Recently, it was reported that in A23187-stimulated Sf9 cells, the D43N or D93N or D43N/D93N cPLA$_2$.$\alpha$ mutants were unable to translocate to the nuclear envelope, whereas the D93N mutant was able to do so, revealing a distinctive role of the two aspartate residues (67). Whether mutations in either of the two aspartate residues would alter the interaction with vimentin is now under investigation.

P60 is Identical to Vimentin—In order to identify P60, the nuclear matrix-rich fraction of 3Y1 cells was separated by SDS-PAGE and electrotransferred to a polyvinylidene difluoride membrane. The P60 protein band was cut off, and peptide mapping using lysylendopeptidase by the method of Cleveland et al. (38) was performed to determine the amino-terminal amino acid sequences of two major fragments. On the basis of these sequences (ARVEVEEDLXED and GNEDLERQ), degenerate oligonucleotides were designed, and degenerate PCR analysis was carried out using 3Y1 cell cDNA as the template. The DNA sequence of a 1.5-kilobase pair fragment thus obtained was determined and found to be identical to the corresponding part of rat vimentin cDNA. A survey of the amino acid sequence of vimentin revealed that the two peptide sequences of P60 that we determined corresponded to amino acids 168–180 and 335–343 of vimentin.

To establish whether P60 is indeed identical to vimentin, we used a mutant clone of SW13, a human adrenocortical carcinoma cell line spontaneously devoid of vimentin expression (39). P60 was detected in the wild-type SW13 cells, which expressed vimentin as an intermediate filament component, but not in the vimentin-deficient clone (Fig. 3A, left panel). Furthermore, immunoblotting with an anti-vimentin antibody showed that vimentin migrated as a 60-kDa protein, which was like P60) detected in 3Y1, 293, and wild-type SW13 cells but not in vimentin-deficient SW13 cells (Fig. 3A, right panel). Moreover, when the membrane shown in Fig. 1F was reprobed with an anti-vimentin antibody, a 60-kDa immunoreactive vi-
malignant-interactive protein in these cells was detected by far-Western analysis. Neither the rod nor the tail domain bound to GST-cPLA2 (1–138) to recombinant His6-tagged vimentin on far-Western analysis. The established stable transformant SW13-G5, which expressed vimentin, and its parental cells, which did not (Fig. 6A), were prelabeled with [3H]AA, and [3H]AA release in response to A23187 was evaluated. SW13-G5 cells released about twice as much [3H]AA as the parental cells (0.52 and 1.2% AA release by parental and G5 cells, respectively) after stimulation with 10 μM A23187 for 30 min (Fig. 6B). The cPLA2 expression levels of these cells, assessed by enzyme assay, were comparable (data not shown). These observations indicate that vimentin expression facilitates cPLA2-mediated AA release.

Our finding that vimentin interacted with cPLA2α via its head domain led us to formulate the hypothesis that the overexpressed vimentin head domain would compete with endogenous vimentin for binding to cPLA2α, thereby exerting a dominant-inhibitory effect on cPLA2α function. We addressed this issue by establishing a 3Y1 cell transfectant that stably overexpressed EGFP-fused vimentin (1–125) (3Y1-vim (1–125)). The expression of vimentin (1–125) in the transfectants was verified by immunoblotting using an anti-EGFP antibody (Fig. 7A). The amount of [3H]AA released (Fig. 7B) and PGE2 produced concomitantly (Fig. 7C) after stimulation with 3 μM A23187 for 30 min by 3Y1-vim (1–125) cells were significantly lower than those produced by the parent 3Y1 cells. The cPLA2α expression levels of both cells were comparable (data not shown). Therefore, vimentin (1–125), which interacts with cPLA2α but lacks the ability to polymerize (41), exerted a dominant-negative effect on cPLA2α function, thereby suppressing AA release and PG generation. It should be noted that there is a significant quantitative difference between decreases in AA (2-fold) and PGE2 (5-fold) caused by overexpressed vimentin head domain. A possible explanation for this is that a PLA2α(s) other than cPLA2α may also contribute to AA release, but only the AA released by cPLA2α can be processed to the PGE2-biosynthetic pathway. Indeed, remaining AA release was partially suppressed by bromoenol lactone (data not shown), which inhibits iPLA2, a PLA2 isozyme playing more important role in phospholipid remodeling than in eicosanoid generation (68, 69). Alternatively, vimentin may function as a scaffold protein for cPLA2α and downstream enzymes (COX and PGE2 synthase) that assists their functional coupling, leading to efficient conversion of a subpopulation of AA to PGE2.

**DISCUSSION**

As has been shown by a number of studies, there is no doubt that cPLA2α regulates the initial step of AA metabolism in response to stimuli that mobilize intracellular Ca2+ (1–8, 12–
In vitro studies employing cPLA₂-α-specific inhibitors (19, 34, 35), antisense oligonucleotides (20, 43), and cPLA₂-α overexpression (33, 44) have shown that this particular PLA₂ isozyme, which bears multifunctional domains, represents a rate-limiting step for the initiation of the lipid mediator-biosynthetic pathway. Furthermore, the importance of cPLA₂-α has been confirmed by in vivo studies on cPLA₂-α knockout mice (45, 46). Following agonist-stimulated transmembrane signaling that increases the cytoplasmic Ca²⁺ level, cPLA₂-α undergoes translocation from the cytosol to the perinuclear envelope and endoplasmic reticulum (12, 13), where many of the downstream eicosanoid-biosynthetic enzymes, including COX-1, COX-2, 5-LO, 5-LO-activating protein, and several terminal PG and leukotriene synthases, are located (27–29). Phosphorylation by mitogen-activated protein kinases increases the intrinsic activity of cPLA₂-α in vitro (8) and synergizes with the Ca²⁺ signaling pathway to provide the optimal AA-releasing response in vivo (6). The C2 domain of cPLA₂-α is essential for Ca²⁺-dependent association of cPLA₂-α with phospholipid vesicles (4, 47, 48), which is facilitated in the presence of phosphatidylinositol 4,5-bisphosphate and, therefore, consistent with the presence of a putative pleckstrin homology domain in the middle part of the enzyme (24). However, the molecular mechanism whereby cPLA₂-α is directed to the intracellular membrane compartments, the perinuclear membranes in particular, in activated cells remains largely obscure.
Depending on the changes in the cytoplasmic Ca$^{2+}$, negative regulator of cPLA$_2$, the functional analysis revealed that p11 inhibited cPLA$_2$ activity both in vitro and in vivo, suggesting that it acts as a negative regulator of cPLA$_2$. In this study, we found that cPLA$_2$ interacted with a major intermediate filament protein, vimentin, which is expressed abundantly in the perinuclear regions of mesenchymal cells and a variety of cultured cells. This interaction occurred between the C2 domain of cPLA$_2$ and the head domain of vimentin. In agreement with the dependence of their interaction on Ca$^{2+}$, confocal microscopic analysis revealed that they colocalized around the perinuclear area in A23187-stimulated but not unstimulated cells. Most importantly, overexpression of full-length vimentin augmented cPLA$_2$-initiated AA metabolism, whereas that of the vimentin head domain exerted a dominant-negative effect. Collectively, these results suggest that vimentin represents a functional adapter for cPLA$_2$ that determines the intracellular localization and thereby modifies the function of cPLA$_2$, depending on the changes in the cytoplasmic Ca$^{2+}$ levels during cellular activation.

The C2 domain of cPLA$_2$, like those of protein kinase C and synaptotagmin, behaves as a Ca$^{2+}$-dependent lipid-binding domain. Recent crystal structural analysis revealed that the C2 domain of cPLA$_2$ captured two Ca$^{2+}$ ions at one end of the domain between three loops, CBR1, CBR2, and CBR3 (36, 37), and the Ca$^{2+}$ ions interacted directly with the phosphate moiety of a lipid head group. In this study, we showed that Asp$^{43}$ (which resides in CBR1) and Asp$^{83}$ (which resides in CBR3) in the C2 domain are involved in the cPLA$_2$-vimentin interaction. This is an important finding, because mutation of either of these two aspartates in the native C2 domain reduced Ca$^{2+}$ binding, eventually leading to reduce phospholipid binding and enzyme activity (37). The cPLA$_2$ C2 domain consists of eight β-sheet structures, β1–β8 (36, 63). Our present findings (that cPLA$_2$ (1–81), which contains the first four β-sheets (β1–β4) of the C2 domain, interacted with vimentin, whereas neither cPLA$_2$ (1–35), which contains the first β-sheet (β1), nor cPLA$_2$ (36–81), which contains β2–β4, did so) suggest that the structural determinants that lie in the β1-β4 region are critical for recognition by vimentin. Interestingly, critical residues required for phospholipid membrane binding are separated from the β1-β4 region (64), raising a possibility that cPLA$_2$ C2 domain could bind simultaneously to both vimentin and phospholipid membranes in a Ca$^{2+}$-dependent manner. If this hypothesis is correct, cPLA$_2$ associated with vimentin could efficiently hydrolyze phospholipids adjacent to vimentin intermediate filaments. In this regard, vimentin would act as a scaffold protein that assists appropriate interaction of cPLA$_2$ with perinuclear phospholipid membranes.

Several lines of evidence have suggested that vimentin intermediate filament plays a role in intracellular lipid transport (50, 51, 65, 66). One function of the cytoskeleton is to organize the subcellular architecture and topography of the organelles (52). Microtubules maintain the organization of the Golgi apparatus and mitochondria are aligned along the microtubules, vimentin intermediate filament forms a cage around developing lipid droplets (53), lipid droplets in adrenal cells are attached to vimentin intermediate filament (54), and the nuclei of vimentin-deficient SW13 cells have an abnormally lobulated shape (55). A number of proteins that regulate cellular response and homeostasis, including kinases (56), heat shock protein (57), and transglutaminase-related antigen (58), bind to intermediate filament. Our results suggest that vimentin intermediate filament may contribute to bringing the proteins involved in AA metabolism into proximity, enabling eicosanoid generation to proceed in the microcompartments.

Our finding that the vimentin head domain alone exhibited a dominant-inhibitory effect on cPLA$_2$ function suggests that the intrinsic activity of cPLA$_2$ does not increase as a result of associating with the vimentin head domain and that the inability of the truncated vimentin to form a filament structure, which depends entirely on the rod domain (40, 41), may prevent proper localization of cPLA$_2$. Serine/threonine residues in the head domain of vimentin are known to be phosphorylated by several protein kinases in vitro and in vivo (59, 60), and such site-specific phosphorylation by various types of protein kinase contributes to regulation of the filament structure. During cytokinesis, the GTP-binding protein Rho-associated kinase phosphorylated Ser$^{7}$ on vimentin at the cleavage furrow in late mitotic cells (61). Ca$^{2+}$ waves in astrocytes induced global phosphorylation of vimentin by Ca$^{2+}$/calmodulin-dependent kinase II (62), and a small population of vimentin intermediate filaments highly phosphorylated by Ca$^{2+}$/calmodulin-dependent kinase II underwent structural alteration into short filaments (62). It remains unclear whether phosphorylation of the vimentin head domain affects the interactions and functions of cPLA$_2$.

Our results shed light on the regulatory mechanism of cPLA$_2$ in immediate AA release, which is accompanied by a sharp increase in the cytoplasmic Ca$^{2+}$ level. Evidence is accumulating to suggest that cPLA$_2$ is also essential for the delayed PG-biosynthetic response induced by proinflammatory cytokines and lipopolysaccharide, despite the relatively poor Ca$^{2+}$ response during this phase. We propose that spatially and temporally synchronous coupling between local elevations in the Ca$^{2+}$ concentration and phosphorylation may converge on the prolonged activation of cPLA$_2$, leading to delayed AA release. Whether vimentin participates in regulation of the delayed response is now under investigation. Nevertheless, the
cooperative action of cPLA2α, an initiator of AA metabolism, and vimentin, an intermediate filament protein, implies that the assembly and/or disassembly of the cytoskeleton components not only play pivotal roles in cell morphology but also affect the cellular capacity to elicit eicosanoid-biosynthetic responses.

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