Roles of Catalytic Domain Residues in Interfacial Binding and Activation of Group IV Cytosolic Phospholipase A2*

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Group IV cytosolic phospholipase A2 (cPLA2) has been shown to play a critical role in eicosanoid biosynthesis. cPLA2 is composed of the C2 domain that mediates the Ca2+-dependent interfacial binding of protein and the catalytic domain. To elucidate the mechanism of interfacial activation of cPLA2, we measured the effects of mutations of selected ionic and hydrophobic residues in the catalytic domain on the enzyme activity and the membrane binding of cPLA2. Mutations of anionic residues located on (Glu419 and Glu420) or near (Asp436, Asp439, and Asp440) the active site lid enhanced the affinity for cPLA2 for anionic membranes, implying that the electrostatic repulsion between these residues and the anionic membrane surface might trigger the opening of the active site. This notion is further supported by a biphasic dependence of cPLA2 activity on the anionic lipid composition of the vesicles. Mutations of a cluster of cationic residues (Lys541, Lys543, Lys544, and Arg488), while significantly enhancing the activity of enzyme, abrogated the specific activation effect by phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2). These data, in conjunction with cell activity of cPLA2 and mutants transfected into HEK293 cells, suggest that the catalytic residues form a specific binding site for PtdIns(4,5)P2 and that the specific PtdIns(4,5)P2 binding is involved in cellular activation of cPLA2. Also, three hydrophobic residues at the rim of the active site (Ile399, Leu400, and Leu552) were shown to partially penetrate the membrane, thereby promoting membrane binding and activation of cPLA2. Based on these results, we propose an interfacial activation mechanism for cPLA2 which involves the removal of the active site lid by non-specific electrostatic repulsion, the interdomain hinge movement induced by specific PtdIns(4,5)P2 binding, and the partial membrane penetration by catalytic domain hydrophobic residues.

Phospholipases A2 (PLA2) catalyze the hydrolysis of membrane phospholipids, the products of which can be transformed into potent inflammatory lipid mediators, platelet-activating factor and eicosanoids that include prostaglandins, thromboxanes, leukotrienes, and lipoxins. Among multiple forms of PLA2 found in mammalian tissues, calcium-dependent group IV PLA2 (cPLA2) has been shown to play a key role in the biosynthesis of inflammatory lipid mediators (1–3). cPLA2 is composed of the amino-terminal C2 domain and the carboxyl-terminal catalytic domain. Previous structural (4) and functional (5, 6) studies have demonstrated that the C2 domain is involved in the membrane binding and subcellular targeting of the cPLA2 molecule. However, the role of the catalytic domain in the membrane binding and activation of cPLA2 is still poorly understood. A recent crystal structure of cPLA2 revealed some unique structural features of the protein that provide a clue to the potential role of the catalytic domain in the interfacial activation of cPLA2 (7). First, the C2 and the catalytic domains are connected by a narrow and flexible linker region (see Fig. 1), implying that a hinge motion of this linker might be involved in cPLA2 activation. Second, the active site entry of the enzyme is partially blocked by a largely amphiphilic lipid (residues 413–457) that is flanked by highly flexible lid hinges (see Figs. 1 and 2). Thus, the interfacial activation of cPLA2 should involve the removal of the lid from the active site. Third, several anionic and cationic patches are present on the putative membrane-binding surface of the catalytic domain that surrounds the active site cavity (see Fig. 1). In particular, two clusters of anionic residues are present in the active site lid and in the lid hinges, respectively, whereas two prominent cationic patches are found in the molecular periphery (see Figs. 1 and 2). Finally, several hydrophobic residues are located at the rim of the active site (Fig. 2).

This study was undertaken to investigate the roles of the catalytic domain in membrane binding and activation of cPLA2. In vitro mutational analyses of ionic and hydrophobic residues in the catalytic domain using surface plasmon resonance, monolayer penetration, and activity assays, in conjunction with the studies of HEK293 cells transfected with cPLA2 and selected mutants tagged with enhanced green fluorescence protein (EGFP), provide new insights into the mechanism of cPLA2 activation and the roles of catalytic domain residues in this process.

EXPERIMENTAL PROCEDURES

Materials—1,2-Di-O-hexadecyl-sn-glycero-3-phosphocholine (DHPC) was from Sigma. 1,2-Di-O-hexadecyl-sn-glycero-3-phosphoglycerol (PG), phosphatidylglycerol; PS, phosphatidylserine; PtdIns(3)P, phosphatidylinositol 3-phosphate; PtdIns(3,4)P2, phosphatidylinositol 3,4-bisphosphate; PtdIns(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PS, phosphatidylserine; cPLA2, phospholipase A2; PyArPC, 1-(1-pyrenedecyl)-2-arachidonoyl-sn-glycero-3-phosphocholine; sPLA2, secretory PLA2; HEK, human embryonic kidney; CHAPS, 3-(3-cholamido-propyl)dimethylammonio)-1-propanesulfonic acid; PH, pleckstrin homology.

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(DHPC) was prepared from DHPC by phospholipase D-catalyzed transphosphatidyltransferase as described (8). 1,2-Bis(12-lipoyloxy)-dodecanoyl-sn-glycero-3-phosphoglycerol (BLPG) and 1-O-(1-pyreneedecyl)-2-arachidonyl-sn-glycero-3-phosphocholine (PyArPC) were synthesized as described previously (9). Phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) was from Amersham Bioscience, phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) was generous gifts from Dr. Karol Bruzik of University of Illinois, Chicago. Phospholipid concentrations were determined by phosphate analysis.

Expressed, Purification of cPLA₂—Baculovirus transfer vectors encoding the cDNAs of cPLA₂ with appropriate catalytic and phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) were generated as a function of time. Typically, the Δσ value reached a maximum after 30 min. The maximal Δσ value depended on the protein concentration and reached a saturation value (i.e. 1 μg/ml). Protein concentrations in the subphase were therefore maintained above such values to ensure that the observed Δσ represented a maximal value. Δσ versus σₜ plots were constructed from these measurements.

Cell Culture, Transfection, and Protein Production—EGFP was cloned from the vector pEGFP (CLONTECH) by PCR with its stop codon removed. It was inserted between the HindIII and the NotI site of a modified pIND vector (Invitrogen) to create the plasmid pIND/EGFP. For subcloning of cPLA₂ and its mutants into the pIND/EGFP vector, NotI and BglII sites were inserted into the 5' and 3' ends, respectively, of corresponding pVL1392-cPLA₂ vectors by PCR. The PCR product was digested and ligated into pIND/EGFP plasmid to create an amino-terminal EGFP fusion protein with a spacer sequence, MRPH. Plasmid DNA for transfection was prepared using an EndoFree Plasmid Maxi kit (Qiagen, Valencia, CA) to avoid possible endotoxin contamination. For protein expression, Sf9 cells were grown to 2 × 10⁶ cells/ml in 350-mI suspension cultures and infected with high titer recombinant baculovirus at a multiplicity of infection of 10. The cells were then incubated for 3 days at 27°C. For harvesting, cells were centrifuged at 1000 × g for 10 min and resuspended in 35 ml of extraction buffer (20 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 10 mM CaCl₂, 0.1% Triton X-100, 100 mM Na₂CO₃, 0.1% CHAPS, and 1 mM phenylmethylsulfonyl fluoride), and CHAPS were from Sigma. Octyl glucoside was from Fisher. Human embryonic kidney cell line (HEK293), Zeocin, and Ponasterone A were from Invitrogen.

Mutagenesis, Expression, and Purification of cPLA₂—Baculovirus transfer vectors encoding the cDNAs of cPLA₂ with appropriate catalytic and phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) were generated as a function of time. Typically, the Δσ value reached a maximum after 30 min. The maximal Δσ value depended on the protein concentration and reached a saturation value (i.e. 1 μg/ml). Protein concentrations in the subphase were therefore maintained above such values to ensure that the observed Δσ represented a maximal value. Δσ versus σₜ plots were constructed from these measurements.

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A stable HEK293 cell line expressing the edysone receptor/invitrogon fusion was used for all cell studies. Briefly, cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in 5% CO₂ and 98% humidity until 90% confluent. Cells between the 5th and 20th passage were passaged into 8 wells of a Lab-Tech™ chambered coverslip. For transfection, these cells were exposed to 150 μl of unsupplemented DMEM containing 0.5 μg of endotoxin-free DNA and 1 μl of LipofectAMINE™ reagent (Invitrogen). Transfection medium was removed; the cells were washed once with FBS-supplemented DMEM and overlaid with FBS-supplemented DMEM containing Zeocin™ (Invitrogen) and 140 μg/ml Ponasterone A to induce protein production.

Confocal Microscopy—Images were obtained using a 4-channel Zeiss LSM 510 laser scanning confocal microscope. EGFP was excited using the 488-nm line of an argon/krypton laser. A 505-nm line pass filter and a ×63, 1.2 numerical aperture water immersion objective were used for all experiments. All experiments were carried out at the same laser power, which was found to induce minimal photobleaching over 30 scans, and at the same gain and offset settings on the photomultiplier tube. Cells for imaging were selected based on their initial intensity. The LSM 510 imaging software was used to control the time intervals for imaging calcium-dependent translocation of cPLA₂. Immediately before imaging, induction media were removed, and the cells were washed twice with 150 μl of 2 mM EGTA and then overlaid with 150 μl of HEK buffer (1 mM HEPES, pH 7.4, containing 2.5 mM MgCl₂, 1 mM NaCl, 0.6 mM KCl, 0.67 mM t-glucose, 6.4 mM sucrose) containing 2 mM EGTA and 10 μM ionomycin. After initial imaging of the cells, 150 μl of HEK buffer containing 6 mM CaCl₂ was added to transfected cells to induce the calcium-dependent translocation of cPLA₂ and its mutants. Control experiments were done with dimethyl sulfoxide in place of ionomycin.

Cellular AA Release—Although the expression of cPLA₂ and mutants in HEK293 cells was induced as described above, cells were radiolabeled with 0.1 μCi/ml [³H]AA (200 Ci/mmol). After 20 h at 37°C,
RESULTS

Mutations of Anionic Residues in the Catalytic Domain—The x-ray crystal structure of cPLA₂ (7) indicates the presence of two clusters of anionic residues in the lid blocking the active site and the hinge region that connects the lid to the rest of the molecule, respectively (see Figs. 1 and 2). Glu⁴¹⁸, Glu⁴¹⁹, and Glu⁴²⁰ on the lid are surface-exposed in the structure, and the Asp⁴³⁶, Asp⁴³⁸, Asp⁴³⁹, and Glu⁴⁴⁰ in the hinge region are also expected to be exposed although their coordinates have not been defined due to high flexibility. Because all intracellular membranes, including perinuclear membranes, contain considerable amounts of anionic phospholipids (15), the docking of cPLA₂ to these membranes by the C2 domain would create energetically unfavorable contacts between the membrane surface and the catalytic domain. This suggests that the interfacial activation of cPLA₂, i.e. the removal of the lid from the active site, could be triggered by the electrostatic repulsion. To test this notion, we mutated these surface anionic residues to lysines, i.e. D₄₃₆K/D₄₃₈K/D₄₃₉K/E₄₄₀K. For Glu⁴¹⁸ and Glu⁴²₀, E₄₁₉A/E₄₂₀A mutation was selected over E₄₁₉K/E₄₂₀K due to low stability of the latter. We then measured the membrane binding affinities of these mutants for zwitterionic (DHPC) and anionic vesicles (DHPG) by SPR analysis. DHPG was selected over its phosphatidylserine (PS) derivative due to difficulty involved in the synthesis of latter. We also measured the enzyme activities of wild type and mutants toward zwitterionic [¹⁴C]SAPC vesicles and anionic mixed vesicles of PyArPC (1 mol %)/BLPG. We previously showed that PyArPC is selectively hydrolyzed in PyArPC/BLPG mixed vesicles because of extremely low activity of cPLA₂ on BLPG (9, 10). As summarized in Table I, cPLA₂ wild type has ~4-fold higher affinity for PC vesicles than for PG vesicles. As expected, E₄₁₉A/E₄₂₀A and D₄₃₆K/D₄₃₈K/D₄₃₉K/E₄₄₀K had wild type-like affinities for zwitterionic PC vesicles. Similarly, the mutations yielded activities toward [¹⁴C]SAPC vesicles within a factor of 2 for wild type. Modestly decreased activities are likely to reflect minor local conformational changes that interfere with the activation process. When the affinity for anionic PG vesicles was measured, the quadruple mutant D₄₃₆K/D₄₃₈K/D₄₃₉K/E₄₄₀K showed 5-fold higher affinity than wild type; as a result, it binds PG vesicles slightly better than PC vesicles. The large increase in affinity for anionic vesicles by the charge reversal indicates that the hinge region containing the Asp⁴³₆/Asp⁴³₈/Asp⁴³⁹/Glu⁴⁴₀ cluster would make energetically unfavorable contact with the anionic membrane surface as cPLA₂ approaches the membrane. Interestingly, D₄₃₆K/D₄₃₈K/D₄₃₉K/E₄₄₀K was only 60% more active than wild type toward PyArPC/BLPG mixed vesicles. This is much lower than expected from its enhanced affinity for PG vesicles, even after taking into account a partial loss of enzyme activity caused by the mutation (see above). The lower-than-expected activity suggests that electrostatic attraction between cationic residues introduced to the lid hinge region of cPLA₂ and the anionic membrane surface interferes with the enzyme activation process. This in turn supports the notion that interfacial activation of cPLA₂ involves the removal of the active site lid by the electrostatic repulsion. On the other hand, E₄₁₉A/E₄₂₀A had a modest but definite 30% gain in affinity for PG vesicles and a slight 10% decrease in activity toward PyArPC/BLPG vesicles. These changes are much smaller than those seen with D₄₃₆K/D₄₃₈K/D₄₃₉K/E₄₄₀K, largely because E₄₁₉A/E₄₂₀A has a two-site charge removal in lieu of a four-site charge reversal in the latter mutant. The data still support the notion, however, that the two lid anionic residues unfavorably interact with the anionic membrane surface.

Mutations of Cationic Residues in the Catalytic Domain—Although the full-length cPLA₂ has ~4-fold higher affinity for PC vesicles than for PG vesicles, this preference is significantly less than that of the isolated C2 domain that strongly prefers PC vesicles (16, 17). Furthermore, it has been reported that cPLA₂ can tightly bind anionic phosphatidylserine vesicles (18) or PtdIns(4,5)P₂-containing PC vesicles (19) even in the absence of calcium. These data suggest that the catalytic domain favors interactions with anionic membrane surface, independently of its calcium-dependent, PC-favoring C2 domain. Consistent with this notion, the catalytic domain of cPLA₂ contains a number of surface-exposed cationic residues, form-
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Table I
Relative activities and affinities of cPLA₂ ionic site mutants

| Enzyme              | SAPC  | PyArPC/BLPG | PC affinity, Kd | PG affinity, Kd |
|---------------------|-------|-------------|-----------------|-----------------|
| Wild type           | 1     | 1           | (4.2 ± 0.3) x 10⁻⁹ | (1.6 ± 0.4) x 10⁻⁸ |
| E419A/E420A         | 0.8   | 0.9         | (4.0 ± 0.5) x 10⁻⁹ | (1.2 ± 0.2) x 10⁻⁸ |
| D436K/D438K/D439K/E440K | 0.6   | 1.6         | (4.0 ± 0.7) x 10⁻⁹ | (3.2 ± 0.5) x 10⁻⁹ |
| K271A/K273A/K274A   | 1.1   | 0.3         | (4.3 ± 1.5) x 10⁻⁹ | (3.0 ± 0.5) x 10⁻⁹ |
| R467A               | 0.9   | 0.6         | (4.0 ± 0.5) x 10⁻⁹ | (3.2 ± 0.7) x 10⁻⁹ |
| K541A/K543A/K544A   | 2.3   | 2.6         | (4.2 ± 0.4) x 10⁻⁹ | (1.2 ± 0.3) x 10⁻⁹ |
| R488E               | 1.2   | 1.5         | (4.5 ± 0.6) x 10⁻⁹ | (1.4 ± 0.3) x 10⁻⁹ |

* Absolute specific activity values for wild type were 100 ± 15 and 20 ± 4 nmol/mg min for SAPC and PyArPC/BLPG vesicles, respectively.

It has been long known that some anionic phospholipids can increase cPLA₂ activities (20). In particular, PtdIns(4,5)P₂ was shown to specifically activate cPLA₂ (19). However, the origin of this activation is not fully elucidated. To investigate this aspect systematically, we measured the enzyme activity and membrane affinity of cPLA₂ and selected mutants in the presence of different anionic phospholipids in mixed vesicles with PC. First, we measured the effect of PG on the vesicle affinity and the enzyme activity of cPLA₂. As expected from the PG-facilitated activation of cPLA₂ that increases monotonously with the increase in PG composition. As the DHPG composition in [14C]SAPC/DHPG-mixed vesicles increased, the enzyme activity measured in terms of [14C]AA release steeply rose up to 90% at 7 mol % PG then decreased with the further increase in PG composition. This unique PG dependence suggests that PG enhances the activity of cPLA₂ not by increasing its membrane affinity but by facilitating its activation process at the membrane surface, presumably by triggering the removal of anionic lipids from the active site by electrostatic repulsion. The sharp bell-shape dependence is likely to reflect the combination of the PG-facilitated activation of cPLA₂ that reaches the plateau at ~7 mol % of PG and the monotonous PG-dependent decrease of membrane affinity. To preclude the possibility that the observed effect is a PG-specific artifact, we performed a similar measurement with PS. As is the case with PG, cPLA₂ has been shown to have lower affinity for PS than for PC (19). Due to the difficulty involved in the synthesis of a non-hydrolyzable PS derivative in large quantity, we used DPPS toward which cPLA₂ shows low activity (i.e. <1% activity toward SACP) and measured the enzyme activity as a function of the PS composition in SACP/DPPS-mixed vesicles. As illustrated in Fig. 3, the PS dependence of cPLA₂ activity was essentially the same as the PG dependence. These data thus indicate that the biphasic dependence of cPLA₂ activity on anionic lipids is common to those anionic lipids for which cPLA₂ has lower affinity than for PC.

We then measured the effects of different phosphoinositides, PtdIns(3)P, PtdIns(3,4)P₂, and PtdIns(4,5)P₂ on cPLA₂ activity toward [14C]SAPC vesicles. It was reported that PtdIns(4,5)P₂ greatly activates cPLA₂ especially at low calcium.

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Fig. 3. Anionic lipid dependence of cPLA₂ activity and affinity. Relative enzyme activity of cPLA₂ toward [14C]SAPC/DHPG (or DPPS)-mixed vesicles was measured as a function of DHPG (●) or DPPS (▼) composition. The enzyme concentration was 15 nM in 20 mM HEPES buffer, pH 7.5, containing 100 mM KCl and free 0.5 mM calcium. Also, relative affinity (ratio of 1/Kₐ) of cPLA₂ for DPPC/DPPG-mixed vesicles was measured as a function of DHPG composition (□) by SPR analysis in the same buffer solution. Each data point represents the average of triplicate measurements.

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References S. Das and W. Cho, unpublished observations.
concentrations (19). We also found that with 10 μM Ca^{2+}, PtdIns(4,5)P_2 specifically activates cPLA_2, up to 3.5-fold at 5 mol % of PtdIns(4,5)P_2 in SAPC vesicles, whereas PtdIns(3)P and PtdIns(3,4)P_2 up to 5 mol % had negligible effect (see Fig. 4A). We did not measure the effect of these phosphoinositides beyond 5 mol %, as higher concentrations would be physiologically irrelevant. When measured by SPR analysis, 5 mol % PtdIns(4,5)P_2 in DHPC vesicles did not significantly enhance the affinity of cPLA_2 (data not shown), indicating that the activity increase is not due to enhanced affinity. To account for this specific effect of PtdIns(4,5)P_2, it was proposed that cPLA_2 has a PH domain-like structure in the region surrounding the Lys^{271}/Lys^{272}/Lys^{274} anionic cluster (19), which was later disputed on the basis of the x-ray structure of cPLA_2 (7). To explore the possibility that any of the two prominent cationic clusters are involved in PtdIns(4,5)P_2 binding, we measured the enzyme activity of cationic site mutants, K271A/K273A/K274A, K541A/K543A/K544A, and R488E, in the presence of PtdIns(4,5)P_2. As shown in Fig. 4B, K271A/K273A/K274A was as active as the wild type toward SAPC vesicles (also see Table I), and its activity increased with the increase of PtdIns(4,5)P_2 concentration (up to 300%), confirming that this cationic cluster is not involved in PtdIns(4,5)P_2 binding. In contrast, neither K541A/K543A/K544A nor R488E was activated by PtdIns(4,5)P_2. In fact, PtdIns(4,5)P_2 modestly reduced the activities of K541A/K543A/K544A and R488E. This suggests that these residues, Arg^{488}, Lys^{541}, Lys^{543}, and Lys^{544}, might form a specific binding site for PtdIns(4,5)P_2.

**Cellular Membrane Translocation and Activation**—To assess the physiological relevance of the effect of PtdIns(4,5)P_2 on cPLA_2 activation, we transiently transfected HEK293 cells with cPLA_2 wild type and two mutants, K271A/K273A/K274A and K541A/K543A/K544A, tagged with EGFP at their carboxyl termini. We then determined their cellular activities by monitoring the PH domain release from radiolabeled cells and their subcellular localization by time-lapse confocal imaging. It has been shown that calcium induces the perinuclear translocation of cPLA_2 (21, 22). As shown in Fig. 5, cPLA_2 was evenly dispersed in the cytoplasm when cells were incubated with a Ca^{2+}-depleted medium and moved to the perinuclear region upon cell activation with external Ca^{2+} and ionomycin, showing a clear annular pattern. Under this condition, HEK293 cells overexpressing wild type cPLA_2 had ~6-fold higher AA releasing activity than parent cells when compared at 30 min after stimulation (see Fig. 6). K271A/K273A/K274A and K541A/K543A/K544A showed the wild type-like subcellular location patterns (see Fig. 5). When compared with the wild type, K271A/K273A/K274A had only 40% of net activity (i.e. corrected for the control background), which compares well with its 30% wild type activity toward PyArPC/BLPG vesicles. This suggests that perinuclear membranes of HEK293 cells, including nuclear envelope, contain a considerable amount of anionic lipids. Interestingly, K541A/K543A/K544A, which was ~2.5-fold more active than wild type in *in vitro* assays with both SAPC and PyArPC/BLPG, was only as active as the wild type in the cellular AA assay. In this regard, it is noteworthy that the wild type cPLA_2 and K541A/K543A/K544A have comparable *in vitro* activity in the presence of 5 mol % PtdIns(4,5)P_2 in SAPC vesicles (see Fig. 4B). Thus, it is possible that perinuclear membranes contain a high enough concentration of PtdIns(4,5)P_2 to fully activate cPLA_2 when it is targeted to these membranes in response to the rise in intracellular calcium concentration. To test this possibility, we measured the effect of the PH domain of phospholipase C-δ1, which was shown to have high specificity for PtdIns(4,5)P_2 (23), on the cellular cPLA_2 activity. When the PH domain of phospholipase C-δ1 tagged with EGFP in its carboxyl terminus was overexpressed, it was localized in both the inner plasma membrane and the perinuclear region (see Fig. 5). Thus, it is evident that PtdIns(4,5)P_2 is present in both the plasma membrane and the perinuclear region of HEK293 cells. We then doubly transfected HEK293 cells with both cPLA_2 (or K541A/K543A/K544A) and the PH domain. As shown in Fig. 6, the AA releasing activity of cPLA_2 was greatly reduced by the co-transfection with the PH domain. The AA release from the doubly transfected cells was only twice higher than that from parent cells (i.e. 6.5-fold drop in net activity). The reduced activity could be due to either specific PtdIns(4,5)P_2 depletion by the PH domain or nonspecific competition for perinuclear membrane-binding sites between the PH domain and cPLA_2 (or a combination of
affinity. Because Ile399 and Leu400 are contiguous, we made a double-site mutant for these residues. As shown in Table II, L552A showed 2-fold reduction in activity should reflect the nonspecific inhibition by the PH domain. This in turn indicates that the depletion of PtdIns(4,5)P₂ by the PH domain is responsible in large part for the 6.5-fold decrease in cPLA₂ inhibition by the PH domain. Most importantly, the selective inhibitory effect of PH domain on the wild type cPLA₂ corroborates the notion that Lys⁵⁴¹, Lys⁵⁴³, and Lys⁵⁴⁴ are involved in PtdIns(4,5)P₂ binding and also indicates that PtdIns(4,5)P₂ plays a significant role in cellular activation of cPLA₂ under the experimental conditions used herein.

Mutations of Hydrophobic Residues in the Catalytic Domain—The rim and the wall of which are made of mainly hydrophobic residues and also contains ionic (primarily cationic), hydrophobic, and aromatic residues on its membrane-binding surface (25). Structure-function studies of various sPLA₂s have shown that their membrane binding is mainly driven by long range electrostatic interactions between surface cationic residues and the anionic membrane surface (26) and complex interactions between aromatic residues and phospholipid head groups (27, 28). Neither gross conformational changes nor a significant degree of membrane penetration by sPLA₂ is involved in their membrane binding.

DISCUSSION

The membrane binding mechanism of secretory PLA₂s (sPLA₂s) has been extensively studied (24). In general, an sPLA₂ has a wide open active site, the rim and the wall of which are composed of mainly hydrophobic residues and also contains ionic (primarily cationic), hydrophobic, and aromatic residues on its membrane-binding surface (25). Structure-function studies of various sPLA₂s have shown that their membrane binding is mainly driven by long range electrostatic interactions between surface cationic residues and the anionic membrane surface (26) and complex interactions between aromatic residues and phospholipid head groups (27, 28). Neither gross conformational changes nor a significant degree of membrane penetration by sPLA₂ is involved in their membrane binding.

FIG. 5. Subcellular translocation of cPLA₂ and mutants upon calcium stimulation. Transiently transfected HEK293 cells were washed twice with 2 mM EGTA and overlaid with 150 µl of HEK buffer containing 2 mM EGTA and 10 µM ionomycin (see “Experimental Procedures”). Cells were then activated by the addition of 150 µl of HEK buffer containing 6 mM calcium. The images were taken before (top row) and 5 min after (bottom row) the calcium stimulation. For the PH domain from phospholipase Cβ-1, transfected HEK293 cells were washed, overlaid with 150 µl of HEK buffer, and imaged.

FIG. 6. Ionomycin-induced AA release from HEK293 cells transfected with cPLA₂ and mutants. Transiently transfected, [³H]AA-labeled HEK293 cells were treated 500 µl of DMEM, 10% FBS medium containing 10 µM ionomycin for 30 min at 37 °C. The [³H]AA release is expressed in terms of the percentage of total radioactivity incorporated into cells. Each value represents the average of triplicate or more determinations.
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TABLE II

Relative activities and affinities of cPLA₂ hydrophobic site mutants

| Enzyme             | Relative activity^a | PC affinity | PG affinity, K_d |
|--------------------|---------------------|-------------|-----------------|
|                    | SAPC   | Pyrene-PC/BLPG | k_a  | k_d  | K_d  |          |
| Wild type          | 1      | 1              | (4.5 ± 0.1) x 10^5 | (2.1 ± 0.2) x 10^-3 | (4.4 ± 0.3) x 10^-9 | (1.6 ± 0.4) x 10^-8 |
| L552A              | 0.35   | 0.47           | (3.9 ± 0.6) x 10^-5 | (3.6 ± 0.3) x 10^-3 | (7.7 ± 1.5) x 10^-9 | (3.8 ± 0.7) x 10^-9 |
| I399A/I400A        | 0.09   | 0.09           | (3.9 ± 0.2) x 10^-5 | (3.0 ± 0.5) x 10^-3 | (1.0 ± 0.2) x 10^-8 | (4.0 ± 1.0) x 10^-8 |

^a Absolute specific activity values for wild type were 100 ± 15 and 20 ± 4 nmol/mg min for SAPC and pyrene-PC/BLPG vesicles, respectively.

As is the case with sPLA₂, the catalytic domain of cPLA₂ contains two prominent cationic patches and some hydrophobic residues on its putative membrane-binding surface. However, the catalytic domain of cPLA₂ is distinct from sPLA₂ in that its active site is partially covered by a lid containing multiple anionic residues. The presence of lid blocking the entry to the active site in the membrane-free state of enzyme strongly suggests that upon membrane binding gross conformational changes of the enzyme might take place to remove the lid from the active site and allow a phospholipid molecule to enter the active site, as is the case with the interfacial activation of lipases (29). The lid (residue 413–457) is made of a short α-helix and a short turn that are flanked by hinges (i.e., residues 408–412 and 434–456) both of which are not defined in the x-ray structure due to high mobility. The external face of the lid contains three anionic residues (Glu418/Glu419/Glu420), whereas a hinge region possesses four anionic residues (Asp436/Asp438/Asp549/Glu540), most of which are expected to be surface-exposed. The presence of these anionic clusters on the membrane-binding surface of cPLA₂ acts on intracellular membranes containing some degree of anionic lipids implies that the removal of active site lid might be triggered by the electrostatic repulsion at the membrane surface. The notion that the interfacial activation of cPLA₂ is triggered by electrostatic repulsion is supported by several findings. First, the mutations of these anionic residues enhance the affinity for anionic vesicles, indicating that indeed these residues unfavorably interact with anionic membranes. Second, the unexpectedly low activity of D436K/D438K/D439K/E440K on PtdArPC embedded in anionic BLPG vesicles suggests that the electrostatic repulsion between the anionic membrane surface and these anionic residues is involved in the removal of the lid from the active site. Third, cPLA₂ activity exhibits a unique bell-shaped dependence of on the PG (and PS) composition of PC/PG(S)-mixed vesicles, despite the monotonously negative effect of PG on the membrane affinity of cPLA₂. It should be noted, however, that cPLA₂ shows relatively high activity on purely zwitterionic vesicles (i.e., maximal activation by PG is 90%), indicating that the active site lid could be removed even in the absence of anionic phospholipids in the membrane. This is presumably because the lid might be non-specifically removed to avoid the desolvation penalty associated with bringing highly anionic residues close to the membrane (30). Thus, it would seem that the lid removal by PG and other non specific anionic lipids is not absolutely required for cPLA₂ activation but that it facilitates the activation under physiological conditions.

Unlike nonspecific anionic lipids (e.g., PG and PS), PtdIns(4,5)P₂ appears to activate cPLA₂ in a specific manner. It was shown that cPLA₂ binds with high affinity and specificity to PtdIns(4,5)P₂ in a 1:1 stoichiometry (19). We found that 5 mol % PtdIns(4,5)P₂ in SAPC vesicles enhances the cPLA₂ activity by 3.5-fold, with a minimal effect on vesicle affinity. This degree of activation is much less than the reported value (>100-fold increase in activity) (19), which might be due to different experimental conditions (i.e., different calcium concentrations and vesicle versus mixed micellar systems). The crystal structure of cPLA₂ did not reveal a well defined PH domain-like structure in the catalytic domain (7). Our mutational analysis suggests, however, that the cationic cluster composed of Lys⁵₄¹, Lys⁵₄₃, Lys⁵₄₄, and Arg⁴₈₈ forms at least a part of PtdIns(4,5)P₂-binding site. Although this part of catalytic domain does not have the PH domain-like structure, it should be noted that a phosphoinositide can be specifically recognized by different structural modules, as evidenced by the specific binding of PtdIns(3)P by FYVE (31) and phox (PX) domains (32). Thus, it is possible that the PtdIns(4,5)P₂-binding site in cPLA₂ might belong to a yet unidentified phosphoinositide-binding motif. Unexpectedly high enzyme activities of two mutants, K541A/K543A/K544A and R488E, raise an interesting possibility about the mechanism by which PtdIns(4,5)P₂ specifically activates cPLA₂. Obviously, these cationic residues do not participate in nonspecific interactions with the anionic membrane surface, as their mutations did not influence the binding to anionic PG vesicles. Instead, it would seem that they are somehow involved in keeping the enzyme in an inactive conformation at the resting state. As shown in Fig. 1, the C2 domain of cPLA₂ also has clustered cationic residues (Arg⁵²⁷, Lys⁵⁵⁸, Arg⁵⁵⁹, and Arg⁵⁶¹) that are separated from the Lys⁵⁴¹/Lys⁵⁴³/Lys⁵⁴⁴/Arg⁴₈₈ cluster by ≥13 Å over the molecular groove. By taking into the account the flexibility of molecular linker region con-
The isolated C2 domain of cPLA2 has been extensively characterized by various biophysical techniques (17, 34, 35). Based on crystal structure of the whole cPLA2 molecule, however, it is difficult to explain how the active site of the catalytic domain is juxtaposed to the membrane while docking the C2 domain to the membrane in the orientation that was suggested by the studies of isolated C2 domain. Interestingly, the hypothetical hinge motion of the linker region induced by PtdIns(4,5)P2 binding can bring the active site closer to the membrane surface with the C2 domain bound to the membrane in its optimal orientation (see Fig. 8).

It has been well established that cPLA2 translocates to and acts on perinuclear membranes in response to a rise in calcium concentration (21, 22). This calcium-dependent subcellular targeting has been ascribed to the C2 domain that has high preference for PC (16, 17, 36) that is abundant in perinuclear membranes (37–39). Our cell data using EGFP-tagged cPLA2 wild type and mutants indicate that perinuclear membranes of HEK293 cells contain a significant amount of anionic lipids (estimated from the low activity of K271A/K273A/K274A), particularly PtdIns(4,5)P2, in high enough concentration to fully activate cPLA2 upon its membrane translocation (estimated from the comparable activities of wild type and K541A/K543A/K544A). PtdIns(4,5)P2 has been found mainly in the plasma membrane (40), but our confocal imaging of the PH domain of phospholipase C-8 shows its presence in the perinuclear region of HEK293 cells. A selective inhibitory effect of the PH domain on cPLA2, with a much lesser degree of inhibition for the PtdIns(4,5)P2-independent K541A/K543A/K544A mutant, strongly suggests that the cellular cPLA2 activity can be regulated by the spatiotemporal dynamics of PtdIns(4,5)P2 in perinuclear membranes. Although it was proposed that PtdIns(4,5)P2 might be able to activate cPLA2 in a calcium-independent manner, calcium-independent cPLA2 activity was extremely low even in the presence of >5 mol % PtdIns(4,5)P2 in our in vitro and cell measurements, indicating that PtdIns(4,5)P2 would regulate cPLA2 activity in concert with calcium rather than independently of calcium. Undoubtedly, further studies are necessary to elucidate this important aspect of cPLA2 activation.

In contrast to sPLA2s, the membrane binding of which does not involve the penetration of hydrophobic residues into the membrane, the membrane binding of cPLA2 involves the calcium-dependent membrane penetration (10) that is mainly mediated by the C2 domain (17). Our monolayer penetration data show that the isolated catalytic domain also has lower but definite membrane penetrating capability, which appears to be attributed at least in part to the hydrophobic residues located in the rim of the active site. The effects of mutations of these hydrophobic residues on membrane binding and activity indicate that they might play some role in elongating the membrane residence time of cPLA2 and perhaps properly orienting the active site at the membrane surface to allow facile movement of a substrate molecule to the active site.

On the basis of these results, we propose a model for the membrane binding and activation of cPLA2 illustrated in Fig. 8. In this model, cPLA2 in the resting state exists as an inactive conformation because of active site lid and the electrostatic repulsion between cationic patches in the C2 domain (Arg57/ Lys61/Arg61/Arg65) and in the catalytic domain (Lys344/Lys543/Lys544/Arg468). Upon the increase of calcium concentration, the C2 domain drives the cPLA2 molecule to the membrane surface. This initial binding involves the membrane penetration of aliphatic and aromatic residues in the calcium-binding loops of the C2 domain and the interaction of cationic residues in the catalytic domain (Lys327/Lys523/Lys724/Arg467) with anionic phospholipids (Fig. 8A). The binding would also bring the anionic residues located in the active site lid and hinge regions close to the anionic membrane surface. The resulting electrostatic repulsion swings the lid away from the active site. How-

**Fig. 8. A hypothetical model for the interfacial binding of cPLA2**. A, calcium ions (brown) drive the membrane translocation of the C2 domain (orange ribbon), which brings anionic residues (red) located in the active site lid and hinge regions in contact with the anionic membrane surface. The electrostatic repulsion removes the lid away from the active site. However, the active site and the residues at the active site rim (magenta) are not juxtaposed to the membrane (cyan, only a monolayer is shown) in this binding mode. B, a PtdIns(4,5)P2 molecule (green) migrates into the groove and specifically interacts with the cationic residues (blue) in the catalytic domain and in the C2 domain. This binding induces the conformational change of cPLA2 that brings its active site closer to the membrane surface. These two types of conformational changes lead to the interfacial activation of the enzyme and allow a substrate molecule to enter the active site for catalytic turnover.
ever, this type of membrane binding cannot lead to the optimal docking of the active site to the membrane surface as long as cPLA$_2$ exists in its inactive conformation. Only after the conformational change that is induced by specific interactions between a PtdIns(4,5)P$_2$ molecule and a group of cationic residues in the catalytic domain (Lys$_5^{44}$/Lys$_5^{44}$/Lys$_5^{44}$/Arg$_{486}$), the active site of cPLA$_2$ is juxtaposed to the membrane (Fig. 8B). These two types of conformational changes, i.e. the removal of active lid by nonspecific electrostatic repulsion and the interdomain hinge movement by specific PtdIns(4,5)P$_2$ binding, expose hydrophobic residues at the rim of the active site and allow them to make energetically favorable hydrophobic interactions with membranes by partially penetrating the hydrophobic core of the membranes. Eventually, these processes allow a substrate molecule to enter the active site for catalytic turnover. It should be noted that all our studies were performed with partially phosphorylated proteins and that the effect of phosphorylation is not taken into account in the current model. In view of the fact that the major phosphorylation site of cPLA$_2$ (Ser$_{505}$) is located in the interdomain hinge region, it is tempting to assume that the effect of its phosphorylation (i.e. introduction of negative charges) might be similar to that of the PtdIns(4,5)P$_2$ binding. Further studies on the interplay of phosphorylation and PtdIns(4,5)P$_2$ binding would provide an important clue to the understanding of the complex cellular membrane targeting and activation mechanism of cPLA$_2$.

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REFERENCES

1. Leslie, C. C. (1997) J. Biol. Chem. 272, 16709–16712
2. Bonventre, J. V., Huang, Z., Taberi, M. R., O’Leary, E., Li, E., Moskwitz, M. A., and Sapirstein, A. (1997) Nature 390, 622–625
3. Uozumi, N., Kume, K., Nagase, T., Nakatani, N., Ishii, S., Tashiro, F., Komagata, Y., Maki, K., Ikuta, K., Ouchi, Y., Miyazaki, J., and Shimizu, T. (1997) Nature 390, 618–622
4. Periasw, O., Fong, S., Lynch, D. E., Bycroft, M., and Williams, R. L. (1998) J. Biol. Chem. 273, 1596–1604
5. Nalefski, E. A., Sultzman, L. A., Martin, D. M., Kria, R. W., Towler, P. S., Knopf, J. L., and Clark, J. D. (1994) J. Biol. Chem. 269, 18239–18249
6. Gijon, M. A., Spencer, D. M., Kaiser, A. L., and Leslie, C. C. (1999) J. Cell Biol. 145, 1219–1232
7. Dessen, A., Tang, J., Schmidt, H., Stahl, M., Clark, J. D., Seehra, J., and Somers, W. S. (1999) Cell 97, 349–360
8. Comfuris, P., and Zwaal, R. F. A. (1977) Biochim. Biophys. Acta 488, 36–42
9. Cho, W., Wu, S. K., Yoon, E., and Lichtenbergova, L. (1999) Methods Mol. Biol. 118, 17–27
10. Lichtenbergova, L., Yoon, E. T., and Cho, W. (1998) Biochemistry 37, 14128–14136
11. Kim, K. P., Raftler, J. D., Bittova, L., Han, S. K., Shintke, Y., Munoz, N. M., Leff, A. R., and Cho, W. (2001) J. Biol. Chem. 276, 11126–11134
12. Stahelin, R. V., and Cho, W. (2001) Biochemistry 40, 4672–4678
13. Cho, W., Bittova, L., and Stahelin, R. V. (2001) Anal. Biochem. 296, 153–161
14. Medcalf, M., and Cho, W. (1998) Biochemistry 37, 4892–4900
15. White, D. A. (1973) in The Phospholipid Composition of Mammalian Tissues (Ansell, G. B., Hawthorne, J. N., and Dwason, R. M. C., eds), 2nd Ed., pp. 441–482, Elsevier Science Publishing Co., Inc., New York
16. Nalefski, E. A., McDonagh, T., Somers, W., Seehra, J., Falke, J. J., and Clark, J. D. (1998) J. Biol. Chem. 273, 1365–1372
17. Bittova, L., Sumandea, M., and Cho, W. (1999) J. Biol. Chem. 274, 9665–9672
18. Hixon, M. S., Ball, A., and Gelb, M. H. (1998) Biochemistry 37, 8516–8526
19. Mostor, M., Six, D. A., and Dennis, E. A. (1998) J. Biol. Chem. 273, 2184–2191
20. Leslie, C. C., and Channon, J. Y. (1990) Biochim. Biophys. Acta 1045, 261–270
21. Glover, S. de Carvalho, M. S., Bayburt, T., Jonas, M., Chi, E., Leslie, C. C., and Gelb, M. H. (1995) Biochemistry 34, 15359–15367
22. Evans, J. H., Spencer, D. M., Zweidach, A., and Leslie, C. C. (2001) J. Biol. Chem. 276, 30150–30160
23. Ferguson, K. M., Lemmon, M. A., Schlessinger, J., and Sigler, P. B. (1995) Cell 83, 1037–1046
24. Gelb, M. H., Cho, W., and Wilton, D. C. (1999) Curr. Opin. Struct. Biol. 9, 428–432
25. Scott, D. L., and Sigler, P. B. (1994) Adv. Prot. Chem. 45, 53–88
26. Han, S. K., Yoon, E. T., Scott, D. L., Sigler, P. B., and Cho, W. (1997) J. Biol. Chem. 272, 3573–3582
27. Sumandea, M., Das, S., Sumandea, C., and Cho, W. (1999) Biochemistry 38, 16290–16297
28. Han, S. K., Kim, K. P., Koduri, R., Bittova, L., Munoz, N. M., Leff, A. R., Wilton, D. C., Gelb, M. H., and Cho, W. (1999) J. Biol. Chem. 274, 11881–11888
29. Cygler, M., and Schrag, J. D. (1997) Methods Enzymol. 284, 3–27
30. Murray, D., and Honig, B. (2002) Mol. Cell 9, 145–154
31. Dumas, J. J., Merithew, E., Sudharsan, E., Rajamani, D., Hayes, S., Lawe, D., Corvera, S., and Lambricht, D. G. (2001) Mol. Cell 8, 947–958
32. Brany, J., Karathanassis, D., Pacold, C. M., Pacold, M. E., Elsen, C. D., Anderson, K. E., Butler, J. L., Lavenir, I., Periasw, O., Hawkins, P. T., Stephens, L., and Williams, R. L. (2001) Mol. Cell 8, 829–839
33. Riza, J., and Sudhof, T. C. (1998) J. Biol. Chem. 273, 15879–15882
34. Nalefski, E. A., and Falke, J. J. (1998) Biochemistry 37, 17642–17650
35. Ball, A., Nielsen, R., Gelb, M. H. (1995) J. Biol. Chem. 270, 15359–15367
36. Brany, J., Karathanassis, D., Pacold, C. M., Pacold, M. E., Elsen, C. D., Anderson, K. E., Butler, J. L., Lavenir, I., Periasw, O., Hawkins, P. T., Stephens, L., and Williams, R. L. (2001) Mol. Cell 8, 829–839
37. Brecher, M. S. (1972) Nat. New Biol. 266, 11–12
38. Daum, G. (1985) Biochim. Biophys. Acta 822, 1–42
39. Williams, S. D., Hsu, F. F., and Ford, D. A. (2000) J. Lipid Res. 41, 1585–1595
40. Martin, T. F. (1998) Annu. Rev. Cell Dev. Biol. 14, 231–264
41. Nicholls, A., Sharp, K. A., and Honig, B. (1991) Proteins 11, 281–296