The cytosolic Group IVA phospholipase A2 (GIVAPLA2) translocates to intracellular membranes to catalyze the release of lysophosphatidic acid and arachidonic acid. GIVAPLA2 translocation and subsequent activity is regulated by its Ca\(^{2+}\)-dependent phospholipid binding C2 domain. Phosphatidylinositol 4,5-bisphosphate (PI-4,5-P\(_2\)) also binds with high affinity and specificity to GIVAPLA2, facilitating membrane binding and activity. Herein, we demonstrate that GIVAPLA2 possessed full activity in the absence of Ca\(^{2+}\) when PI-4,5-P\(_2\) or phosphatidylinositol 3,4,5-trisphosphate were present. A point mutant, D43N, that is unable to bind Ca\(^{2+}\) also had full activity in the presence of PI-4,5-P\(_2\). However, when GIVAPLA2 was expressed without its Ca\(^{2+}\)-binding C2 domain (\(\Delta C2\)), there was no interfacial activity. GIVAPLA2 and \(\Delta C2\) both had activity on monomeric lysophospholipids, \(\Delta C2\), but not the C2 domain alone, binds to phosphoinositides (PIP\(_s\)) in the same manner as the full-length GIVAPLA2, confirming the location of the PIP\(_s\) binding site as the GIVAPLA2 catalytic domain. Moreover, proposed PIP\(_s\)-binding residues in the catalytic domain (Lys\(^{488}\), Lys\(^{541}\), Lys\(^{543}\), and Lys\(^{544}\)) were confirmed to be essential for PI-4,5-P\(_2\)-dependent activity increases. Exploiting the effects of PI-4,5-P\(_2\), we have discovered that the C2 domain plays a critical role in the interfacial activity of GIVAPLA2 above and beyond its Ca\(^{2+}\)-dependent phospholipid binding.

The Group IVA phospholipase A2 (GIVAPLA2) plays a central role in intracellular phospholipid hydrolysis. Although it is only one of many different mammalian phospholipase A\(_2\) (1), it is the rate-limiting provider of lysophospholipid and the free polyunsaturated fatty acids such as arachidonic acid that go on to form platelet-activating factor and eicosanoids, respectively (2–5). These various downstream products are central to many physiological processes as well as many pathological conditions (6,7).

The activity of GIVAPLA2 in mammalian cells is regulated by at least two major mechanisms that can act separately or in conjunction with each other. The first is by increasing intracellular Ca\(^{2+}\) concentrations, which leads to the translocation of GIVAPLA2 from the cytosol to its substrate phospholipids in the Golgi, ER, and nuclear membranes (8–10). This Ca\(^{2+}\)-dependent process is mediated by the C2 domain of GIVAPLA2, which binds two Ca\(^{2+}\) ions with a low micromolar affinity (11,12). The increased [Ca\(^{2+}\)] that leads to translocation of the C2 domain to membranes also leads to membrane penetration of several hydrophobic side chains (13–16), which allows the catalytic \(\alpha/\beta\) hydrolase domain to come into contact with its phosphatidylinositol substrate (17). The second major regulatory mechanism for GIVAPLA2 is through phosphorylation at one or more serines (18–20). It appears that phosphorylation leads to an activation of GIVAPLA2, by increasing the specific activity of the enzyme (18–20). Besides Ca\(^{2+}\) and phosphorylation, other factors, such as phosphoinositides (PIP\(_s\)), have been implicated in the regulation of GIVAPLA2 activity.

Early reports by Kojima and co-workers (21) and Leslie and Channon (22) on partially purified rat and mouse GIVAPLA2, respectively, indicated that several anionic lipids, especially polyphosphoinositides, increased the activity of GIVAPLA2. We showed with pure, recombinant human protein that GIVAPLA2 activity is generally enhanced by anionic phospholipids but specifically and more potently enhanced by PIP\(_s\), with phosphatidylinositol 4,5-bisphosphate (PI-4,5-P\(_2\)) being optimal (23). We further demonstrated that GIVAPLA2 binds in a 1:1 stoichiometry to PI-4,5-P\(_2\) with high affinity and specificity (23). In the presence of PI-4,5-P\(_2\) and the absence of Ca\(^{2+}\), GIVAPLA2 both bound to phosphatidylinositol-containing surfaces and was active in vitro (23). Extending this work to cellular systems, we demonstrated that elevated levels of both phosphatidylinositol 4-phosphate (PI-4-P) and PI-4,5-P\(_2\) correlated with and were necessary for GIVAPLA2-dependent arachidonate release by lipopolysaccharide-primed, UV light-activated P388D1 murine macrophage-like cells (24,25). Importantly, no change in intracellular [Ca\(^{2+}\)] was detected, further supporting the potential importance of the PIP\(_s\) effect (24). There have now been several reports of GIVAPLA2 activity in vivo without any change in the resting levels of intracellular Ca\(^{2+}\) (5,24–27).

In this study, we expand the understanding of how GIVAPLA2 may be regulated by Ca\(^{2+}\) and PIP\(_s\). We show that GIVAPLA2 has significant Ca\(^{2+}\)-independent activity in the

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‡ To whom correspondence should be addressed: Dept. of Chemistry and Biochemistry, University of California, San Diego, La Jolla, CA 92093-0601.

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presence of many PIP₃s. We have shown that the Asp⁴³ → Asn mutation that cannot bind Ca²⁺ also has full activity in the presence of PIP₃-5,6-P₂. Whereas binding to Ca²⁺ is not necessary for GIVAPLA₂ activity, we now show that the presence of the C2 domain is required for all interfacial activity. This result demonstrates for the first time a second, novel role of the C2 domain, in that it is required to maintain GIVAPLA₂ in an active conformation or orientation in vesicles.

Finally, we have confirmed that the active site domain alone, and not the C2 domain, contains a functional PIP₃ binding site. This site includes four lysine residues at positions 488, 551, 543, and 544.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine brain PI-4,5-P₂ and PI-4-P were from Roche Applied Science. Pure, native human GIVAPLA₂ (28), pure human serum 228 to alamine GIVAPLA₂, (5228A)29, anti-Group IVA PLA₂ antibody (30), and a pALTER plasmid (Promega, Madison, WI) with the cDNA of His-tagged Group IVA PLA₂ (31) were generous gifts from Drs. Ruth Kramer and John Sharp (Li.)lly). Pure, recombinant human GIVAPLA₂ proteins (His₂-tagged wild type and the quadruple Ser to Ala mutant at residues 437, 454, 505, and 727 (32)) were generous gifts from Dr. Michael Gell (University of Washington, Seattle). Pure, recombinant human GIVAPLA₂ proteins (His₂-tagged wild type and the two mutants, K488E and K541A/K543A/K544A (33)) were generous gifts of Dr. Wonhwa Cho (University of Illinois, Chicago). Radiolabeled 1-α-1-palmityl-2-(1-1⁴C)phosphatidylincholine (PAPC) and 1-1⁴C]phosphatidylincholine (Lyso-PC) were provided by PerkinElmer Life Sciences. Dipalmitoyl PI-3-P, PI-3,4-P₂, and a pALTER plasmid (Promega, Madison, WI) with the cDNA of His-tagged Group IVA PLA₂ (31) were generous gifts from Dr. Ching-Shih Chen (The Ohio State University, Columbus, OH). All other cold phospholipids were from Avanti Polar Lipids Inc. (Ala- baster, AL). PIP Strips™ were from Echelon Research, Inc. (Salt Lake City, UT). Glassclad 18 silanizing agent was from United Chemical Technologies, Inc. (Bristol, PA). Biosafe II liquid scintillation mixture was from RPI Corp., Piscataway, NJ. Olighomocytidylate primers were from Froligo (La Jolla, CA). Ph/Turbo DNA polymerase was from Stratagene (La Jolla, CA). EcoRI, XhoI, and DpnI endonucleases were from Invitrogen. BglII was from Amersham Biosciences. Ndel was from New England Biolabs (Beverly, MA). Bacular baculovirus was from United Chemical Technologies, Inc. (Bristol, PA). Biosciences. Immobilon-P membrane was purchased from Millipore Biosciences. Bovine brain PI-4,5-P₂ and PI-4-P were from Roche Applied Science. Immobilon-P membrane was purchased from Millipore Corp. (Bedford, MA).

**Group IVA PLA₂ Cloning and Mutagenesis**—A pALTER vector containing the cDNA of human GIVAPLA₂ with a C-terminal His tag extension (abbreviated as His GIVAPLA₂) was transferred into a baculovirus transfer vector pVL1393 in the proper orientation by double digesting the pALTER-His-GIVAPLA₂ with EcoRI and BglII, isolating the GIVAPLA₂ insert, and ligating it into the precinct pVL1393 plasmid. GIVAPLA₂ has been almost exclusively studied as a homo-dimer (23). The protocols and reagents for generating recombinant GIVAPLA₂ in Spodoptera frugiperda (Sf9) insect cells were from Pharmingen (BD Biosciences) unless otherwise indicated. In brief, Sf9 insect cells from suspension culture (EX-CELL™ 400 with Glu) were plated and co-transfected with Bacularogel linearized baculovirus DNA and baculovirus transfer vectors containing either His GIVAPLA₂, D43N, N129C, the C2 domain, or the seven other point mutants. In order to be certain to obtain the correct, pure protein, plaque assays were performed to clonally select and amplify one virus that was confirmed to express the active, folded, and correctly sized protein. The plaque assay was performed as indicated (Pharmingen BD Biosciences), but the plaques were more readily visualized with Neutral Red Dye (see Supplemental Protocol (Clontech BD Biosciences)). After a clonal virus was obtained and amplified, insect cell tissue culture plates (20 cm) were infected at a multiplicity of infection of <1. Recombinant protein was harvested from the infected Sf9 cells that were grown in TNM-FH insect cell medium.

**GIVAPLA₂ Construct Purification**—Sf9 cells that had been infected with recombinant baculoviruses were pelleted and then lysed with Pharmingen’s insect cell lysis buffer on ice for 60 min and then centrifuged for 15 min at 4 °C at 16,000 × g to remove all unbroken cells and debris. This clarified lysate contained substantial levels of recombinant His GIVAPLA₂ (or control XYLE protein), such that the recombinant protein band was easily visible and distinguishable on an SDS-PAGE, followed by Coomassie-stained gel. Activity assays confirmed the high levels of expression.

Purified, recombinant protein was easily obtained from the clarified lysate using His tag affinity purification. The Talon system (Clontech/BD Biosciences) containing a Co⁵⁺ resin was successfully used according to instructions for batch adsorption purification. The recombinant, tagged proteins were found to elute successfully from the Talon resin with imidazole elution buffer but not by low pH elution buffer. The lysate, washes, and all elutions were subject to SDS-PAGE followed by Coomassie staining. Each construct was also subject to a Western blot (on Immobilon-P membranes) and was easily detected with an anti-His tag antibody (data not shown) as a control for further use of the antibodies. The eluate was used in subsequent experiments since the recombinant proteins were essentially pure and at a high concentration after elution by imidazole. The pure, recombinant proteins in imidazole-elution buffer had full activity based on native control protein, such that all GIVAPLA₂-related proteins were used directly from the concentrated elute or after storage at −20 °C in the imidazole elution buffer supplemented with glycerol.

**Standard PAPC PLA₂ Activity Assay**—For the basal specific activity of GIVAPLA₂ (23), assays were performed in buffer composed of 20 mM HEPES at pH 7.7, 100 mM KCl, 200 μM CaCl₂, 1 mg/ml fatty acid free bovine serum albumin (BSA), and 1 mM dithiothreitol. The mixed micelles were composed of 1 mM PAPC (200,000 ppm) and 3 mM Triton X-100. Aliquots of 10 μl were added to 10 μl of a 10 μM solution of the test compound. After 30 min incubation, the reaction was stopped and the samples were analyzed as above.

The mixed micelle substrate was prepared as described previously (23, 37). The micelles were initially made up in 20 mM HEPES and 100 mM KCl (190 μl/assay) to form a cloudy white solution of multilamellar vesicles upon vortexing. The initial substrate buffer did not contain Ca²⁺ to avoid precipitating any phospholipid, especially the PIP₃s, and incorporated 60 μl of Triton X-100 (1 mg/ml) and 40 μl of 150 μM per assay), resulting in a rapid clearing of the cloudy white vesicles as the clear mixed micelles form at a 2.5-fold higher concentration (2.5 μM PAPC and 7.5 μM Triton X-100) than that observed in the final assay mix. The mixed micelles were allowed to form over 30 min (with occasional vortexing) to ensure that all the phospholipids were released from the glass surface and incorporated into the micelles. After micelle formation, the mixed micelles (200 μl/assay) and the assay buffer (250 μl/assay) each were added to glass test tubes (16 × 125 mm). All of the Ca²⁺ or EGTA was found exclusively in the assay buffer. The tubes had been previously siliconized with Glassclad 18, to

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help prevent the enzymes and substrates from adsorbing onto the glass surface. The mixed micelles in the tubes were then gently shaken in a 40 °C water bath for several minutes. The reaction was initiated by the addition of GIVAPLA2 (1 μg in 50 μl per sample of assay buffer that lacked Ca2+ and EGTA) followed by vortexing. This brought the final volume to 500 μl and the desired final concentrations as noted above. After a 60-min incubation, the reaction was quenched, and the fatty acids were extracted using a modified Dole protocol (38) as previously described (23, 37). The final radioactive cpm were doubled in the calculation of specific activity because only 1 of 2 of ml of heptane were counted. Background experiments were where no enzyme was added were always performed and were subtracted from the data obtained with enzyme.

**Standard PI-4,5-P2, PLA2 Activity Assay** — This assay was identical to the standard PACP PLA2 activity assay, with the exception that the mixed micelles contained 1 mol % PI-4,5-P2 (i.e. 0.96 nm PACP (200,000 cpm), 0.04 nm PI-4,5-P2, and 3 nm Triton X-100) (23). Since PI-4,5-P2 enhances the activity of GIVAPLA2 by up to 120-fold, the amount of enzyme used was dropped to 0.1 μg, and the time was shortened to 10 min. The micelles were prepared in an identical manner to that described above. The amount of enzyme and time of incubation were varied in all assays to achieve <5% hydrolysis. This low level of hydrolysis ensured that the substrate-containing interface was not significantly perturbed by the hydrolysis products. The specific activities obtained in the standard assays vary, particularly with different sources of the GIVAPLA2 (native or His-tagged enzyme from our laboratory, Lilly, Dr. Gelb, or Dr. Cho); however, the ratio of the activity with PI-4,5-P2 to without PI-4,5-P2 is remarkably consistent at around 100-fold. In all experiments, a matched wild type control is compared with each mutant to control for the source of the enzymes and the tags they may contain.

**Monomeric and Micellar Lysophospholipase Activity Assay** — The Lyso-PC monomer assay conditions, adapted from previous work (13, 39), were 20 μM HEPES (pH 7.5), 100 mM KCl, 200 μM Ca2+, 1 mg/ml BSA, and 1 mM diethiothreitol in a final volume of 500 μl. The monomer assay contained 4 μM Lyso-PC, which is below the critical micelle concentration of 7 μM (40). The substrate preparation was essentially identical to the above assays, except that the monomers were suspended in assay buffer (400 μl/assay) without any Ca2+ or EGTA. The Ca2+ or EGTA (50 μl) was added directly to the assay tubes before the assay was initiated.

The assay was only begun after confirming the expected cpm per unit volume in the substrate solution. This was important because, in contrast to micelles and vesicles, there were no visual signs of fully solubilized monomers. The Dole procedure was used to extract the fatty acids exactly as described above, except that excess cold palmitic acid (50 μg) was added to the quenched assay tube to enhance the extraction efficiency of the small amounts of pure, radiolabeled palmitic acid produced (~0.03 μg). In the micellar form of this assay (above 7 μM Lyso-PC), the Ca2+ concentration was increased up to 1 mM, and the radioactivity was adjusted (where possible) to 200,000 cpm. For this higher substrate concentration, no unlabeled palmitic acid was needed in the assay workup. Unless otherwise indicated, all results are presented as the mean ± S.D. from a representative experiment with each condition tested in duplicate or triplicate.

**PIP2, Binding Assay** — PIP2, GIVAPLA2 binding was determined using PIP Strips from Echelon Research, Inc., and was carried out according to the provided protocols. A small plastic dish (~66 cm2) for each PIP Strip and a rocking platform kept at 4 °C were used for each of the following steps. The strips were blocked for 1 h in 15 ml of 10 mM Tris (pH 8.0), 150 mM NaCl, 0.1% Tween 20, and 3% BSA (TBS-T/BSA). The target protein was then incubated with a strip overnight (12–18 h) in 10 ml of TBS-T/BSA. The strip was then washed with the standard washing protocol: three washes with 10 ml of TBS-T/BSA for 10 min. The micelles were prepared in an identical manner to that described above. The amount of enzyme and time of incubation were varied in all assays to achieve <5% hydrolysis. This low level of hydrolysis ensured that the substrate-containing interface was not significantly perturbed by the hydrolysis products. The specific activities obtained in the standard assays vary, particularly with different sources of the GIVAPLA2 (native or His-tagged enzyme from our laboratory, Lilly, Dr. Gelb, or Dr. Cho); however, the ratio of the activity with PI-4,5-P2 to without PI-4,5-P2 is remarkably consistent at around 100-fold. In all experiments, a matched wild type control is compared with each mutant to control for the source of the enzymes and the tags they may contain.

**RESULTS**

**Ca2+-independent Activity of GIVAPLA2** — In the absence of Ca2+, GIVAPLA2 is known to bind to and be active on lysophospholipid micelles (41), membrane interfaces containing PI-4,5-P2 (23), and membranes composed of the nonnatural anionic phospholipid phosphatidylethanolamine (32). We have previously shown that in the presence of Ca2+, PIP2s and PIP2 gave larger enhancements of GIVAPLA2 activity than PIPs, which in turn gave larger enhancements than all other anionic phospholipids tested (23). Herein we have successfully analyzed the activity enhancements of PIP2s on pure, human GIVAPLA2 in the absence of Ca2+. As expected, the presence of 5 mol % phosphatidylethanolamine, phosphatidylserine, phosphatidylethanolamine, phosphatidic acid, and phosphatidylglycerol (PI) in PACP/Triton X-100 mixed micelles did not yield any activity for native GIVAPLA2 in the absence of Ca2+ as seen in Fig. 1. However, in the presence of 1 mol % PI-3, P1-4, P1-3,4, P1-4,5-P2, P1-4,5-P2, or PIP2 there was significant GIVAPLA2 activity even in the absence of Ca2+ ([Ca2+] < 2 nm, Fig. 1, gray bars). Moreover, the activities with 1 mol % PI-4,5-P2 and PIP2 were similar to one another with and without Ca2+ and were both more than 100-fold higher than the control that lacked PIP2s but contained Ca2+. Importantly, PI-4-P and P1-3,4-P2 also gave very significant activity enhancements in the absence of Ca2+, relative to PACP alone (with Ca2+). These GIVAPLA2 activities with Ca2+ were only 30 and 16% of the activities with Ca2+, respectively, but they are still far above the undeletable activity of GIVAPLA2 on PACP without Ca2+ or PIP2s.

**PI-4,5-P2, PLA2 Activity Assay with D43N and ΔC2** — The addition of EGTA without any exogenous Ca2+ should have reduced the free [Ca2+] to extremely low levels ([Ca2+] < 2 nm). Nevertheless, we tested recombinant Ca2+-binding-deficient mutants of GIVAPLA2 to confirm that the PI-4,5-P2-GIVAPLA2 interaction can unambiguously replace the Ca2+-C2 domain interaction. A conceptual diagram of these various GIVAPLA2 mutant constructs is shown in Fig. 2.

Using the recombinant, pure D43N, ΔC2, and His GIVAPLA2, we measured activity in the PI-4,5-P2 activity assay with and without Ca2+. The results shown in Fig. 3 clearly demonstrate that in the presence of PI-4,5-P2, D43N has the same activity as His GIVAPLA2. As expected from its inability to bind Ca2+, the D43N activity remained the same with or
FIG. 2. Comparison of His GIVAPLA₂ and major deletion and mutant constructs. His GIVAPLA₂ is shown in a linear schematic with the N-terminal C2 domain containing a critical calcium ligand (Asp²⁴⁵) in orange and the C-terminal αβ hydrolase domain containing the active site dyad (Ser²²⁸ and Asp⁴⁴⁹) in red. Three other constructs are also shown: the independent C2 domain, the independent αβ hydrolase domain (∆C2), and the full-length single site mutant D43N.

FIG. 3. PI-4,5-P₂-enhanced activity of GIVAPLA₂, D43N, and ∆C2 on mixed micelles with and without Ca²⁺. The PI-4,5-P₂ activities of His GIVAPLA₂ and its mutants, D43N and ∆C2, were tested on PI-4,5-P₂-containing mixed micelles composed of Triton X-100 (3 mM), 1-palmitoyl-2-(1-[^1⁴C]arachidonoyl)-PC (0.96 mM, 200,000 cpm), and PI-4,5-P₂ (0.04 mM, 1 mol %). White bars, assays performed with 200 μM Ca²⁺; gray bars, assays performed with 500 μM EGTA ([Ca²⁺] < 2 nM).

FIG. 4. Basal activity of GIVAPLA₂, D43N, and ∆C2 on mixed micelles in the absence of PI-4,5-P₂. The basal activities of His GIVAPLA₂ and its mutants, D43N and ∆C2, were tested on mixed micelles composed of Triton X-100 (3 mM) and 1-palmitoyl-2-(1[^1⁴C]arachidonoyl)-PC (1 mM, 200,000 cpm) in the presence of 200 μM Ca²⁺.

FIG. 5. Lysophospholipase activity of GIVAPLA₂, D43N, and ∆C2 in the absence of PI-4,5-P₂ on lysosphospholipid micelles or monomers. A, the Lyso-PLA activities of His GIVAPLA₂ and its mutants, D43N and ∆C2, were tested on 1-(1[^1⁴C]palmitoyl)-Lyso-PC micelles (100 μM, 200,000 cpm). B, the Lyso-PLA activities of His GIVAPLA₂ and its mutants, D43N and ∆C2, were tested on 1-(1[^1⁴C]palmitoyl)-Lyso-PC monomers (4 μM, 240,000 cpm).

without Ca²⁺. The ∆C2 construct, however, did not have any activity with or without Ca²⁺, in striking contrast to the D43N activity.

PAPC PLA₂ Activity Assay with D43N and ∆C2—Given that the activity of D43N is comparable with His GIVAPLA₂ in the presence of PI-4,5-P₂, it was critical to test D43N without PI-4,5-P₂. As shown in Fig. 4, D43N, ∆C2, and a mixture of ∆C2 and the C2 domain all have no significant activity above base line on PAPC/Triton X-100 mixed micelles in the presence of Ca²⁺. D43N and ∆C2 were not expected to have any activity in these Ca²⁺-dependent assay conditions based on previous reports (13, 42). No activity was seen under these conditions for any His GIVAPLA₂ construct in the absence of both PI-4,5-P₂ and Ca²⁺ (data not shown).

Micellar and Monomeric Lysophospholipase Activity of D43N, ∆C2, and GIVAPLA₂—Previously, we (41) and others (39) had shown that GIVAPLA₂ has Ca²⁺-independent Lyso-PLA activity on pure micelles of 1-palmitoyl-Lyso-PC. We measured the activity of D43N and ∆C2 on Lyso-PC micelles in the absence of PI-4,5-P₂. As shown in Fig. 5A, His GIVAPLA₂ and D43N both have high activity on 100 μM Lyso-PC (relative to ∆C2 and background) at 50 and 15 nmol/min/mg enzyme, respectively. For His GIVAPLA₂ and D43N, there was no difference in activity in the presence or absence of Ca²⁺ (data not shown). The activity of ∆C2 on Lyso-PC micelles was dramatically lower than His GIVAPLA₂ and D43N, indicating that the C2 domain is required for activity on Lyso-PC micelles (Fig. 5A) as well as on PI-4,5-P₂/PAPC/Triton X-100 mixed micelles (Fig. 3).

The lack of interfacial PLA₂ or Lyso-PLA activity for ∆C2 might have indicated that it was incapable of all catalytic activity. Previous studies had shown that ∆C2 lacked the ability to bind to and hydrolyze membranes with or without Ca²⁺ in all tested systems (13, 16). It was also shown, however, that both full-length GIVAPLA₂ and the ∆C2 construct possessed Lyso-PLA activity on monomeric (nonaggregated) substrate (13), since the catalytic residues are all located on this domain (Fig. 2). In order to confirm that ∆C2 behaved as previously reported, a monomer assay was utilized with 4 μM Lyso-PC substrate. As shown in Fig. 5B, His GIVAPLA₂, D43N, and ∆C2 all have significant activity on monomeric Lyso-PC substrate (relative to background and the limit of detection), indicating their active sites are folded properly. Compared with their monomer Lyso-PLA activity (Fig. 5B), the activities of His GIVAPLA₂ and D43N on micellar Lyso-PC substrate were dra-
matically higher at 100 μM (Fig. 5A; 35- and 12-fold, respectively), indicating competent interfacial activity. However, whereas ΔC2 showed a steady increase in activity up to the critical micelle concentration (0.5 nmol/min/mg at ~7 μM), there was no further increase up to 100 μM Lyso-PC (Fig. 5, A and B, and data not shown). In fact, monomer activity accounts for all of the activity (0.54 ± 0.04 nmol/min/mg) seen for ΔC2 in Fig. 5A.

The apparent lower activity of ΔC2 versus His GIVAPLA2 and D43N at 4 μM Lyso-PC is consistent with premicellar aggregation induced by these enzymes. Premicellar aggregation would result in the creation of an interface that could be more efficiently hydrolyzed by the interfacially competent GIVAPLA2 and D43N, but not ΔC2. Whereas the monomer activity of ΔC2 is somewhat lower than the other two full-length enzymes, the hydrolysis measured (0.4 nmol/min/mg) was well above the detection limit at these conditions of 0.075 nmol/min/mg. Some questions have been raised about whether the monomeric Lyso-PLA activity of GIVAPLA2 is due to the same active site as the PLA2 activity. To address this concern, we tested the full-length, active site mutant, S228A. The Ser mutant had no monomeric Lyso-PLA activity. Under the conditions tested, an activity as low as 0.1 nmol/min/mg could have been detected for S228A. The lack of all types of activity by S228A confirms that all known GIVAPLA2 catalytic activities depend on Ser228 (29).

Binding of GIVAPLA2 Constructs to Immobilized Phospholipids—To determine which domain(s) of GIVAPLA2 binds to PIP₅₅₅₅, an overlay blot was performed using nitrocellulose-immobilized PIP₅₅₅₅ on a PIP Strip. The PIP Strips have 100-pmol spots of various natural and synthetic phospholipids affixed to them. This includes synthetic dipalmitoyl compounds of all possible PIP₅₅₅₅. The PIP Strips have been used to study the PIP₅₅₅₅ binding interactions of various PIP₅₅₅₅ antibodies (Echelon Research, Inc.) and PIP₅₅₅₅ proteins (43–45), including phospholipase C (46). The standard PIP Strip binding protocol calls for a 12–16-h incubation with 5 μg of target protein. When this was carried out, no binding was detected for GIVAPLA2 (Fig. 6).

Theoretically, GIVAPLA2 is able to sequentially hydrolyze the sn-2 fatty acyl chain of a phospholipid, followed by the sn-1 fatty acyl chain of the resulting lysophospholipid (39, 47). If this occurred on the PIP Strips, the polar head groups would have been released from the surface (along with any bound GIVAPLA2), eliminating the chemiluminescence signal.

In order to address the possible hydrolysis of the PIP₅₅₅₅ from the PIP Strips, we utilized two complementary techniques. First, GIVAPLA2 was preincubated with an excess of an irreversible serine-dependent PLA2 inhibitor, MAFP. After incubation of MAFP-inhibited GIVAPLA2 with the PIP Strips and probed with anti-GIVAPLA2 antibody, there was significant signal at several PIP₅₅₅₅ spots, as seen in Fig. 6. Second, to rule out any effects of the MAFP, S228A was used. The results for S228A did not differ from those seen with MAFP-treated GIVAPLA2 (data not shown). The location and identity of each spot on the PIP Strips is shown in a schematic diagram in Fig. 6.

The identity of the spots indicated that under these conditions GIVAPLA2 bound PI-3-P, PI-4-P, and phosphatidylinositol 5-phosphate to give a consistently intense signal in every condition tested. Signal was also detected with PI-4,5-P₂, PI-3,4-P₂, phosphatidylinositol 3,5-bisphosphate, and occasionally weak signal with other anionic lipids, PIP₅₅₅₅, PI, phosphatidic acid, and phosphatidylserine. No signal was detected for phosphatidylethanolamine, phosphatidylcholine, or inositol 1,3,4,5-tetraakisphosphate. The same results were obtained for His/MAFP-GIVAPLA₂.ΔC2

Fig. 6. Binding of GIVAPLA₂ and various mutants to PIP₅₅₅₅ in a protein-phospholipid overlay blot. Binding of various GIVAPLA₂ constructs was tested in protein-phospholipid overlay blots (PIP Strips). The PIP Strips were prespotted with 100 pmol of the indicated phospholipids. Each strip was incubated with 5 μg of GIVAPLA₂, MAFP-treated GIVAPLA₂, ΔC2, or the C2 domain and probed with an anti-GIVAPLA₂ antibody or anti-His tag antibody followed by an HRP-conjugated secondary antibody. The location of each phospholipid is as shown on the schematic diagram. The blots correspond to the specific proteins as indicated under each blot. PE, phosphatidylethanolamine; PS, phosphatidylserine; PG, phosphatidyglycerol; PA, phosphatic acid; PI-5-P, phosphatidylinositol 5-phosphate; IP₅₅₅₅, inositol 1,3,4,5-tetraakisphosphate.

GIVAPLA2 with an anti-GIVAPLA2 antibody or the TetraHis antibody (data not shown).

To test which domain was able to bind to PIP₅₅₅₅, ΔC2 and the C2 domain were preincubated with MAFP, incubated with PIP Strips, and probed with the TetraHis antibody. The results clearly indicated that ΔC2, but not the C2 domain, showed the same signal as the MAFP-treated GIVAPLA2 (Fig. 6). In addition, D43N was tested in the same manner and found to match the results seen for the MAFP-treated GIVAPLA2 (data not shown). Although it was crucial for native and His GIVAPLA2, MAFP was not necessary and did not interfere with the results for ΔC2 and the C2 domain. No effect of Ca²⁺ was seen for any construct tested.

The PIP Strip assay was used primarily to compare the ability of different GIVAPLA₂ constructs to bind to PIP₅₅₅₅. For this qualitative comparison among the various constructs, this assay was efficient and straightforward. However, it cannot be used as a quantitative measure of strength of binding or specificity of binding for any protein. There are several reasons for this limitation, including the nonphysiological density of negative charges from the various PIP₅₅₅₅ (especially PIP₅₅₅₅) in the pure spots and the improbable 30-Å² surface area for each polar head group. This surface density calculation would strongly suggest that the phospholipids on the PIP Strips were not arranged as a canonical monolayer on the PIP Strips.

Another factor to consider is the effect of the nonbinding
portions of each protein. Many PIP₃-binding proteins, such as pleckstrin homology domains and antibodies, generally have single phospholipid binding sites in various surface-exposed loops (48, 49). Some of these proteins like pleckstrin homology domains also bind tightly to the soluble head groups, such as inositol 1,4,5-trisphosphate (50). GIVAPLA₂ is different, because it does not bind inositol 1,4,5-trisphosphate, but instead binds to PIP₃s only in an interface (23). Moreover, unlike pleckstrin homology domains, GIVAPLA₂ has at least three membrane binding attachment points: the active site, the C2 domain, and the PIP₃ binding site. When GIVAPLA₂ binds to the PIP Strips, it is conceivable that one or both of the other binding sites are brought in close proximity to the surface, raising the possibilities for steric or electrostatic repulsion. These possibilities might be exacerbated on the PIP Strips by the lack of lateral mobility of the PIP₃s and would be most significant with PIP₃₄⁶⁶₃.

**Confirmation of the Key Residues for the PIP₃ Interaction**—Recent work of Das and Cho (33) indicated using mutagenesis that several basic residues were directly involved in the binding and activity enhancements from PI-4,5-P₂. The two mutants, K488E and K541A/K543A/K544A, were assayed on phospholipid vesicles under conditions where GIVAPLA₂ activity with 5 mol % PI-4,5-P₂ was up to 3.5-fold higher than without (33). Both K488E and K541A/K543A/K544A had increased basal activity (4- and 2-fold, respectively) (33). Interestingly, both of these mutants had no increase in activity with up to 5 mol % PI-4,5-P₂ but rather each had a slight decrease (33). Because of these small enhancements from PI-4,5-P₂ in these assay conditions, complicated by the mutants’ enhanced basal activity, we undertook to confirm the results in assay conditions that maximize the level of enhancement from PI-4,5-P₂ (100-fold or greater) (23).

We tested the basal and PI-4,5-P₂-stimulated activity of K488E and K541A/K543A/K544A in the standard PAPC and standard PI-4,5-P₂ assays. Our results showed that the basal activity increased ~4-fold for both (Fig. 7), which qualitatively agrees with the previous report (33). The results in the presence of only 1 mol % PI-4,5-P₂ showed that K488E and K541A/K543A/K544A activities increased only 15–60%, compared with ~50-fold for the WT control (Fig. 7). These results qualitatively match those seen previously (33). Thus, we have confirmed that these residues appear to be crucial for the GIVAPLA₂-PIP₂ interaction.

Ruling Out Proposed PIP₃-binding Residues—In a previous report (23), we hypothesized that the PIP₃-binding site of GIVAPLA₂ might be located at a string of basic residues between amino acids 271 and 283. We have now mutated all of those basic side chains to remove the charges or functional groups. The mutations included K271Q, K273Q, R274Q, S278A, K281Q, K282Q, and K283Q. The resulting mutant proteins had no defects in basal or PI-4,5-P₂-enhanced activity (data not shown) and thus gave around the same 100-fold increase with 1 mol % PI-4,5-P₂ as seen for WT GIVAPLA₂ in the standard PAPC assay, the quadruple mutant had about 25% of the specific activity of the control His GIVAPLA₂ (Fig. 8). There was no positive or negative interaction between PIP₃-stimulation and GIVAPLA₂ phosphorylation at these four residues, which are normally partially phosphorylated during their cellular expression (36). One other phosphorylation site (Ser²¹⁵) was recently identified (20). This fifth potential phosphorylation target was not phosphorylated in the His GIVAPLA₂ control used in Fig. 8, based on previous detailed research of the same protein (36) and thus is also apparently uninvolved in the effects of PI-4,5-P₂. Importantly, these results suggest that phosphorylation and levels or availability of PIP₃s can coordinate the activity of GIVAPLA₂ (Fig. 8, S228A activity without PI-4,5-P₂ versus GIVAPLA₂ activity with PI-4,5-P₂).

Basal and PI-4,5-P₂-enhanced GIVAPLA₂ Activity Highly Dependent on the Quality of the Interface—The high Lyso-PLA activity of GIVAPLA₂ is a useful feature of GIVAPLA₂ that we have more fully explored. In Triton X-100 mixed micelles, the large GIVAPLA₂ activity enhancements from PI-4,5-P₂ were seen when the substrate was either PAPC or Lyso-PC, as shown in Fig. 9A. For GIVAPLA₂, the Lyso-PLA activity on PI-4,5-P₂/Lyso-PC/Triton X-100 mixed micelles was Ca²⁺-inde-
Novel Role of GIVAPLA2 C2 Domain in Ca\textsuperscript{2+}-independent Activity

The addition of 3 mM Triton X-100 to 1 mM Lyso-PC should have had a surface dilution effect on the activity of GIVAPLA2, reducing the activity 4-fold (56, 57). Indeed, in the presence of Ca\textsuperscript{2+} and absence of PI-4,5-P2, the activity decreases by about 6.5-fold with the dilution of Triton X-100 (Fig. 9, B versus A, white bars). However, when PI-4,5-P2 was incorporated into the Lyso-PC micelles, the expected 4-fold decrease from Triton X-100 dilution was instead a 4-fold increase (Fig. 9, B versus A, gray bars). This indicates that in the presence of Ca\textsuperscript{2+}, the PI-4,5-P2 effects on GIVAPLA2 Lyso-PLA activity depend on the quality of the interface.

The same surface dilution effects on Lyso-PLA activity should be seen in the absence of Ca\textsuperscript{2+} (Fig. 9, A and B, gray bars). However, without PI-4,5-P2, the 4-fold dilution with Triton X-100 gives a 26-fold reduction of activity. This indicates that in the absence of Ca\textsuperscript{2+}, Lyso-PLA activity also strongly depends on the quality of the interface. Finally, in the presence of PI-4,5-P2 with no Ca\textsuperscript{2+}, the dilution by Triton X-100 gives a 1.4-fold increase in contrast to the anticipated 4-fold decrease. These results confirm that the quality of the interface is also important for Ca\textsuperscript{2+}-independent, PI-4,5-P2-dependent activity. Together, these results indicate that the nature or quality of the interface, be it detergent mixed micelles, lysophospholipid micelles, or small unilamellar vesicles, can dramatically impact the activity of GIVAPLA2 as well as the effects of Ca\textsuperscript{2+} and PI-4,5-P2 on its activity. By taking advantage of the effects of Ca\textsuperscript{2+}, PI-4,5-P2, and the quality of interface, a specific assay for GIVAPLA2 was developed (58) that can distinguish this enzyme from all known mammalian PLAA\textsubscript{2}. The specific assay is particularly useful to distinguish GIVAPLA2 activity in samples of crude tissue homogenates or cellular preparations from mouse, rat, and human sources (58).
GIVAPLA2 is normally found evenly distributed throughout the cytosol, whereas its substrate phospholipids are in the intracellular membranes such as ER, Golgi, and nuclear envelope (10). It is well accepted that the translocation of GIVAPLA2 to its substrate membranes can be regulated by intracellular [Ca\(^{2+}\)] (see Ref. 10 and references therein). Whereas the effects of Ca\(^{2+}\) are mediated through the C2 domain, we have now shown that the C2 domain of GIVAPLA2 is apparently required for Ca\(^{2+}\)-independent interfacial activity. This suggests a novel second role for the C2 domain in the Ca\(^{2+}\)-independent activation of the catalytic domain. This second role may also be important for Ca\(^{2+}\)-dependent interfacial activity but would be obscured by the primary, Ca\(^{2+}\)-dependent membrane binding role of the C2 domain.

Recently, evidence has accumulated for the translocation and activation of GIVAPLA2 without a corresponding rise in intracellular [Ca\(^{2+}\)] (5, 24–27). Along with our previous results (23), the results presented herein further strengthen the notion that PI-4,5-P\(_2\), and possibly other PIP\(_n\)s, may have an analogous role to Ca\(^{2+}\) in increasing the membrane affinity of GIVAPLA2. This membrane affinity increase would facilitate increased activity by bringing enzyme and substrate together.

Nonplasma membrane PIP\(_n\)s are synthesized in situ (e.g. nuclear envelope, Golgi, and ER membranes) and have been implicated in a wide variety of functions separate from the plasma membrane (59–62). The Ca\(^{2+}\)-independent activity for GIVAPLA2, seen in vitro at 1 mol % PI-4,5-P\(_2\), is a physiologically relevant surface concentration for many cell membranes (63). More recently, significant levels of PI-4,5-P\(_2\) have been visually identified in various intracellular membranes such as Golgi, ER, and cytosolic nuclear envelope in astrocytoma and squamous carcinoma cells (61) and at the cytosolic perinuclear membranes of HEK293 cells (33). In the HEK293 cells, the perinuclear PI-4,5-P\(_2\) matched the localization seen separately for GIVAPLA2 in the same cells (33). Co-transfection of a PI-4,5-P\(_2\)-binding protein and GIVAPLA2 partly reduced the GIVAPLA2-dependent arachidonic acid release (33), indicating that there may indeed be a physiological interaction between GIVAPLA2 and PI-4,5-P\(_2\).

Herein we report that GIVAPLA2 can have its highest activity on PI-4,5-P\(_2\)- or PIP\(_n\)-containing mixed micelles with or without Ca\(^{2+}\). Along with PI-4,5-P\(_2\) (23) and Lyso-PC (39, 41), PIP\(_n\) is shown to be a third physiologically relevant lipid that can lead to full Ca\(^{2+}\)-independent GIVAPLA2 activity in vitro. However, PI-4,5-P\(_2\), as the much more abundant precursor of PIP\(_n\) (63, 64), is probably more relevant than PIP\(_n\). In addition to PI-4,5-P\(_2\) and PIP\(_n\), PI-3,4-P\(_2\) and PI-4-P also gave significant, but not maximal, Ca\(^{2+}\)-independent activity. Since PI-4-P is the most abundant of the PIP\(_n\) species (24, 63), it is possible that it too could significantly contribute to the activity of GIVAPLA2 in vivo. The up-regulation of PI-4-P levels, and subsequently PI-4,5-P\(_2\) levels, was observed in murine P388D1 macrophage-like cells primed by lipopolysaccharide and activated by UV light (24). Under these conditions, GIVAPLA2 specifically acted to release arachidonic acid without any changes in intracellular [Ca\(^{2+}\)] (24). The increased levels of PIP\(_n\) seen in the P388D1 cells may have been generated at the intracellular membranes to which GIVAPLA2 targets (10), as one or more PI kinases have been observed to localize to those membranes (60, 65).

The full activity of D43N in PI-4,5-P\(_2\) mixed micelles and high activity toward Lyso-PC micelles unambiguously confirmed that native and His GIVAPLA2 can have full activity in the absence of Ca\(^{2+}\). Whereas Ca\(^{2+}\) may be dispensable for GIVAPLA2 activity, the C2 domain is not. The ΔC2 construct did not have any PLA\(_2\) or Lyso-PLA activity on interfacial substrates with or without Ca\(^{2+}\), PI-4,5-P\(_2\), or both. The monomer Lyso-PLA activity of ΔC2 indicated that the catalytic site was functional but lacked any activity on aggregated substrates. In an attempt to rescue the defect in ΔC2, free C2 domain was added, creating a 1:1 ratio between the C2 domain and ΔC2. This mixture did not have any activity (with or without PI-4,5-P\(_2\)), meaning that the two separate domains did not interact with each other to form an interfacially competent enzyme. These results indicate for the first time that an intact C2 domain is required for GIVAPLA2 interfacial activity regardless of its capacity to bind Ca\(^{2+}\).

PI-4,5-P\(_2\)-binding assays were employed to determine whether the C2 domain or the ΔC2 construct (or both) contained the PIP\(_n\) binding site. The PIP\(_n\)-binding pattern seen in the PIP Strip assay for the MAFP-inhibited native GIVAPLA2, His GIVAPLA2, S228A, and D43N all matched ΔC2, whereas the C2 domain showed no PIP\(_n\) binding. This suggested that the active site domain, but not the C2 domain, binds PIP\(_n\).s. These results complement the recent finding that a specific GIVAPLA2 PIP\(_n\) binding site is located on the active site domain (33). We have confirmed these results with our own assay systems and concluded that the PI-4,5-P\(_2\)-binding site resides probably include Lys\(_{488}\), Lys\(_{541}\), Lys\(_{543}\), and Lys\(_{544}\), which are indeed located in the ΔC2 construct (33). Previous results suggest that the catalytic domain may also bind to phosphatidylinmethanol vesicles in a Ca\(^{2+}\)-dependent manner (32), perhaps through the PIP\(_n\) binding site. Interestingly, these four residues are identical in all vertebrate GIVAPLA2 orthologs but not in its paralogs, GIVBLA2 or GIVCPLA2. We have found that these two paralogs are not activated by PI-4,5-P\(_2\), in contrast to GIVAPLA2 (66). The location of the PIP\(_n\) binding site on the GIVAPLA2 catalytic domain contrasts to the recently identified PI-4,5-P\(_2\) binding site in the C2 domain of protein kinase Ca (67).

Although the C2 domain may not be required for PIP\(_n\) binding, it seems to be necessary for a catalytically competent interfacial enzyme, perhaps by facilitating an interdomain conformational activation or active site orientation. One other possibility is that various groups of C2 domain residues are separately involved in PIP\(_n\) binding, Lyso-PC binding, and the traditional Ca\(^{2+}\)-dependent membrane binding. This seems less likely given the presence of the key PIP\(_n\)-binding residues on the catalytic domain and the ability of the catalytic domain itself to bind to PIP\(_n\)s on PIP Strips. The C2 domain has previously been shown to possess some Ca\(^{2+}\)-independent membrane affinity (32, 68), which, while weak, could help explain the critical role of the C2 domain in interfacial activity.

Several others reports have shown that PIP\(_n\)s, including PI-4,5-P\(_2\), enhance the activity of GIVAPLA2 by less than 10-fold (21, 22, 33, 55). At first glance, this seems to contradict our reports that PI-4,5-P\(_2\) enhances the activity of GIVAPLA2 in large unilamellar vesicles composed of PAPC by 20-fold (1 mol %) or 55-fold (3 mol %) and in PAPC/Triton X-100 mixed micelles by up to 120-fold (1 mol %) (23). A likely explanation is that when PI-4,5-P\(_2\) is added to assay systems that have low activity, the enhancing effects are striking, as in Triton X-100 mixed micelles (23). When PI-4,5-P\(_2\) is added to assay systems that already have high activity, the enhancing effects of PI-4,5-P\(_2\) may appear muted as in Lyso-PC micelles (Fig. 9B) and small unilamellar vesicles (21, 22, 33, 55). Nevertheless, despite the high basal levels of activity seen in these reported assays, the additions of small amounts of PI-4,5-P\(_2\) led to reliable, although modest, 4–6-fold activity enhancements as in Fig. 9B and Refs. 22, 33, and 55.

Interestingly, the enhancing effects of PI-4,5-P\(_2\) are not mim-
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