Roles of Acidic Phospholipids and Nucleotides in Regulating Membrane Binding and Activity of a Calcium-independent Phospholipase A2 Isoform

Phospholipase A2 (PLA2) activity plays key roles in generating lipid second messengers and regulates membrane topology through the generation of asymmetric lysophospholipids. In particular, the Group VIA phospholipase A2 (GVIA-iPLA2) subfamily of enzymes functions independently of calcium within the cytoplasm of cells and has been implicated in numerous cellular processes, including proliferation, apoptosis, and membrane transport steps. However, mechanisms underlying the spatial and temporal regulation of these enzymes have remained mostly unexplored. Here, we examine the subset of Caenorhabditis elegans lipases that harbor a consensus motif common to members of the GVIA-iPLA2 subfamily. Based on sequence homology, we identify IPLA-1 as the closest C. elegans homolog of human GVIA-iPLA2 enzymes and use a combination of liposome interaction studies to demonstrate a role for acidic phospholipids in regulating GVIA-iPLA2 function. Our studies indicate that IPLA-1 binds directly to multiple acidic phospholipids, including phosphatidylinerine, phosphatidylglycerol, cardiolipin, phosphatidic acid, and phosphorylated derivatives of phosphatidylinositol. Moreover, the presence of these acidic lipids dramatically elevates the specific activity of IPLA-1 in vitro. We also found that the addition of ATP and ADP promote oligomerization of IPLA-1, which probably underlies the stimulatory effect of nucleotides on its activity. We propose that membrane composition and the presence of nucleotides play key roles in recruiting and modulating GVIA-iPLA2 activity in cells.

The phospholipase A2 (PLA2) superfamily consists of at least 16 distinct groups, all of which share a common enzymatic function (1, 2). Specifically, PLA2 isoforms cleave the sn-2 acyl bond of phospholipids and release two biologically active molecules, a free fatty acid and a lysophospholipid. The released fatty acid can function as a second messenger in cell signaling (3). For example, arachidonic acid liberated from iPLA2-mediated hydrolysis of membrane phospholipids has been shown to alter the activity of ion channels and protein kinases in neurons, which affects their excitability (4, 5). Additionally, arachidonic acid is a precursor for eicosanoid production, which regulates the inflammatory response and other signal transduction events (6). Independently, lysophospholipids can dramatically impact membrane architecture. In particular, an increased local concentration of lysophospholipid density in a lipid bilayer can generate or stabilize membrane curvature by introducing cone-shaped molecules into a relatively flat surface (7).

The GVIA subgroup of the PLA2 superfamily was the first discovered to lack dependence on calcium for function (8). Additionally, all members of this subfamily harbor ankyrin repeats, typically upstream of their lipase domains (9). Studies have shown that a single human gene (iPLA2B) encodes five unique splice variants, three of which exhibit lipase activity (GVIA-1, GVIA-2, and GVIA-3) and two others (GVIA ankyrin-1 and GVIA ankyrin-2) that lack a carboxyl-terminal lipase domain but bind to full-length iPLA2B isoforms and suppress their catalytic activities (10). Two additional GVIA-iPLA2 members (GVIA-4 and GVIA-5) are generated via post-translational, caspase-mediated cleavage but retain lipase activity (11–13).

Mutations in iPLA2B have been genetically linked to a neurodegenerative disorder known as infantile neuroaxonal dystrophy (INAD) (14). Symptoms from INAD typically manifest themselves during early childhood, between 6 months and 3 years of age. Disease progression is rapid, resulting in severe spasticity that impairs normal movement and results in cogni-
ative decline, visual impairment, and ultimately death (15, 16). A major pathologic feature of INAD is the appearance of abnormal nerve endings in both the central and peripheral nervous systems. Large axonal swellings called spheroids, which accumulate membranes with tubulovesicular structure, are common (17, 18). Moreover, spheroid formation is thought to cause axonal degeneration (19). iPLA2β-deficient mice also accumulate axonal spheroids, which have been shown to contain numerous morphologically abnormal mitochondria (20). Additionally, significant axonal membrane degeneration was observed in mutant animals (21). Together, these data suggest that iPLA2β normally functions to maintain membrane architecture at multiple subcellular locations.

Although the pathologic features of INAD appear to be limited to the nervous system, iPLA2β is expressed ubiquitously. In most cell types, the enzyme is largely cytoplasmic (10). However, subsequent to a variety of stimuli, iPLA2β can accumulate in the nucleus, at the endoplasmic reticulum, at the Golgi, and within mitochondria (22–26). How iPLA2β is targeted to these different membranous organelles remains unclear. Here, we examine the family of GVIA-iPLA2 enzymes expressed in Caenorhabditis elegans. Unlike mammalian cells, the C. elegans genome encodes seven putative enzymes that exhibit significant homology to the iPLA2β patatin-like lipase domain, including the consensus motif (GXSPG). We demonstrate that the most highly related iPLA2β homolog in C. elegans (IPLA-1) assembles into an active homo-oligomer in solution, which is stimulated by the presence of ATP or ADP and binds specifically to acidic phospholipids with a relatively high affinity. Moreover, the presence of acidic phospholipids dramatically elevates the specific activity of IPLA-1, suggesting a role for charged lipid headgroups in modulating GVIA-iPLA2 function in cells.

EXPERIMENTAL PROCEDURES

Protein Purification and Hydrodynamic Studies—Recombinant protein expression was performed using Rosetta2 (DE3) Escherichia coli. To enable purification of IPLA-1, a GST tag was appended onto its amino terminus. Specifically, the gene encoding IPLA-1 was amplified from a C. elegans cDNA library and cloned into the bacterial expression construct pGEX6P-1, which encodes a protease cleavage site that enables postpurification removal of the GST tag. Purifications were conducted using glutathione-agarose beads, and untagged IPLA-1 was recovered following treatment with Prescission protease in solution. For size exclusion chromatography, purified IPLA-1 was applied onto a Superose 6 gel filtration column (GE Healthcare), and 1-ml fractions were collected. The Stokes radius of IPLA-1 was calculated from its elution volume, based on the elution profiles of characterized globular standards (27). Similarly, C. elegans embryo extract was separated over the Superose 6 gel filtration column, and fractions were immunoblotted following SDS-PAGE using IPLA-1 antibodies to determine the Stokes radius of endogenous IPLA-1. Glycerol gradients (4 ml; 10–30%) were poured using a Gradient Master and fractionated (200 µl) from the top by hand. Sedimentation values were calculated based on the fractionation profile of characterized standards run on separate gradients in parallel (27). To estimate the molecular weight of protein complexes based on their hydrodynamic properties, we used the Svedberg equation, \( M = 6\pi n \eta a (1 - w) \), where \( M \) is the native molecular weight, \( n \) is the viscosity of the medium, \( \eta \) is Avogadro’s number, \( a \) is the Stokes radius, \( w \) is the sedimentation value, \( v \) is the partial specific volume, and \( r \) is the density of the medium (28). For multi-angle light scattering (MALS) experiments, purified IPLA-1 was applied onto a Wyatt WTC-0305S column, which was coupled to a miniDAWN TREOS three-angle light scattering detector (Wyatt Technology Corp.). Data were collected every second at a flow rate of 0.7 ml/min and analyzed using ASTRA software, which determines molecule weight and mass distribution of samples based on light scattering.

Antibody Production—C. elegans IPLA-1 antibodies were raised in rabbits by immunization (Covance) with a polyhistidine-tagged form of full-length IPLA-1 produced in E. coli. Antibodies were subsequently affinity-purified from serum by binding to a column harboring GST-IPLA-1 as described previously (29).

Production of Liposomes and Co-flotation Assays—Liposomes containing combinations of phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), cardiolipin (CL), phosphatidylglycerol (PG), phosphatidic acid (PA), phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PI4P) and phosphatidylinositol 4,5-bisphosphate (PI4,5P2) were prepared by extrusion through polycarbonate filters with a pore size of 100 nm (Avanti Polar Lipids). For co-flotation assays, 0.5% Rhodamine-PE was incorporated into liposomes to enable normalization subsequent to recovery. Liposomes were first incubated with IPLA-1 in buffer (50 mM Hepes, pH 7.6, and 100 mM NaCl) prior to mixing with Accudenz density medium. Mixtures were overlaid with decreasing concentrations of Accudenz (0–40%) and centrifuged for 2 h at 280,000 × g (30). During this period, liposomes and bound IPLA-1 floated to the buffer/Accudenz interface and were harvested by hand. Recovery of liposomes was normalized based on the fluorescence intensity of the sample, and equivalent fractions (when comparing flotation experiments using liposomes of differing compositions) were separated by SDS-PAGE and stained with Coomassie to determine the relative amount of protein that bound.

IPLA-1 Activity Assay—A mixture of radiolabeled 1-palmitoyl-2-[1-14C]palmitoyl-sn-glycerocephosphocholine \((^{14}C\)-labeled DPPC; 1 mol %) and excess unlabeled DPPC was prepared in the presence of PS, PE, PG, PI, CL, PA, PI4P, or PI4,5P2, dried under a stream of nitrogen and resuspended in assay buffer (5 mM EDTA, 1 mM ATP, 0.4 mM Triton X-100, 2 mM DTT, 100 mM HEpes, pH 7.5) by vortexing and heating (to 40 °C). Lipids were incubated with purified IPLA-1 (500 ng) for 30 min at 40 °C, the reaction was quenched, and free fatty acids were extracted as described previously (31). Isolated fatty acids were analyzed by liquid scintillation counting to quantify IPLA-1 activity. The characterized iPLA2 inhibitors bromoenoic lactone (BEL) (5 µM) and methyl arachidonyl-fluorophosphonate (MAFP) (50 µM) were included individually to verify the specificity of the lipase (32).

Isothermal Titration Calorimetry—All isothermal titration calorimetry experiments were performed on a MicroCal ITC.
200 calorimeter (GE Healthcare). Binding isotherms were recorded following 4-µl injections of lipids (20 mM; 50% PS, 40% PC, and 10% PE) into a cell containing recombinant IPLA-1 (50 mM). Each experiment consisted of 10 injections performed at 2.5-min intervals (27). Background (injections of lipids into a cell containing buffer alone) was subtracted from the binding isotherms, and data were analyzed using a nonlinear least square program (Origin, MicroCal).

RESULTS

The C. elegans Genome Encodes Multiple Putative GVIA-iPLA₂ Isoforms—The largest molecular weight isoform of human iPLA₂ (GVIA-2) harbors multiple ankyrin repeats followed by a patatin-like lipase domain, which utilizes a catalytic serine (Ser-519). Additionally, GVIA-2 contains an ATP binding motif but lacks a domain that associates with calcium (10). The enzyme is sometimes referred to as PNPLA9 because it is a member of the patatin-like phospholipase domain-containing (PNPLA) family. At least eight additional PNPLA enzymes have been identified, and collectively they play numerous important roles in metabolic regulation (33). Based on a combination of characteristics exhibited by GVIA-2 and additional sequence homology, we identified a set of seven putative GVIA-iPLA₂ enzymes in C. elegans, three of which harbor numerous ankyrin repeats, similar to human GVIA-2 (Fig. 1A). We refer to these three proteins as IPLA-1 (C45B2.6), IPLA-2 (F47A4.5), and IPLA-3 (W07A8.2). None of the seven C. elegans proteins exhibited strong sequence homology with other lipases in the PNPLA family, with the exception of the calcium-independent iPLA₂ isoform PNPLA8 (GVIB-iPLA₂). In particular, iPLA-6 (Y73B6B.4) exhibited extensive sequence conservation with PNPLA8 (supplemental Fig. S1A), suggesting that they may function in an orthologous fashion. In contrast, the other six C. elegans proteins exhibited more homology with GVIA-iPLA₂.

Because PLA₂ isoforms play a critical role in the regulation of membrane architecture and organization, which is necessary for normal development, we examined the impact of inhibiting

![FIGURE 1. The C. elegans genome encodes multiple putative GVIA-iPLA₂ isoforms that exhibit redundancy in function. A, schematics depicting the domain organization of putative GVIA-iPLA₂ enzymes in C. elegans. For comparison, a schematic highlighting the characterized domains found in human GVIA-2 is provided (top). The number of amino acids found in each protein (bottom, right) and the percentage of sequence similarity, as compared with the human GVIA-2 patatin-like lipase domain, are also shown. B, the brood sizes of control, ipla-1, ipla-2, ipla-3, ipla-1;ipla-2, ipla-2;ipla-3, and ipla-1;ipla-2;ipla-3 mutant animals were determined within a 24-h period, after animals became fertile. In each case, at least 15 animals were examined, and none demonstrated an egg-laying defect. A significant difference (**) in brood size was observed between control and ipla-2 mutant animals. Similarly, a significant difference in brood size was observed between ipla-2 and ipla-1;ipla-2;ipla-3 mutant animals. **, p < 0.05 (paired t test). Error bars, S.E.]
multiple GVIA members on *C. elegans* reproduction and embryogenesis. Deletion mutations in *ipla-1* (ok1098), *ipla-2* (tm1035), and *ipla-3* (tm1584) have been identified previously, and each is predicted to introduce a premature stop codon, which would truncate the encoded proteins and eliminate their catalytic lipase domains (supplemental Fig. S1B). To determine whether the loss of individual GVIA members impacts reproduction rate and embryo development, we calculated the number of viable progeny that individual animals produced in a 24-h period, after becoming fertile (Fig. 1B). On average, control hermaphrodites incubated at 20 °C produced 61 ± 6 fertilized eggs during the time course, 99.3% of which hatched and developed normally (*n* = 15 animals). Similarly, *ipla-1* and *ipla-3* mutant animals generated 59 ± 6 and 58 ± 6 fertilized eggs, respectively, less than 2% of which failed to hatch in each case (*n* = 15 animals each). In contrast, *ipla-2* mutant animals generated significantly fewer progeny in a 24-h period (48 ± 4 eggs), although 98.5% subsequently hatched (*n* = 15 animals; Fig. 1B). Additionally, larval development was strikingly slower in *ipla-2* mutant animals. Whereas control hermaphrodites required 59 ± 6 h to reach adulthood at 20 °C after hatching, *ipla-2* mutant animals, incubated under identical conditions, required 91 ± 15 h. Nonetheless, we failed to identify significant differences in organelle morphology (including the endoplasmic reticulum, Golgi, endosomal membranes, and plasma membrane), as determined by conducting immunofluorescence studies in fixed embryos lacking IPLA-2 (data not shown). To uncover potential redundancy among the putative lipases, we generated a series of double and triple mutant animals. Only when the function of all three proteins was impacted did we observe a further significant decline in brood size (Fig. 1B). The *ipla-1;ipla-2;ipla-3* triple mutant animals produced only 38 ± 6 eggs in a 24-h period (*n* = 18 animals). This finding suggests that multiple GVIA enzymes function together to regulate reproduction and/or early development. Again, we failed to observe a significant change in embryo viability (96.3% of progeny hatched), and we did not witness defects in organelle architecture (data not shown). Larval development in the triple mutant background continued to be slow, requiring 108 ± 18 h to reach adulthood after hatching, similar to that observed in *ipla-2* single mutant animals. Individually depleting other putative GVIA-iPLA$_2$ isoforms using RNA interference in *ipla-1;ipla-2;ipla-3* triple mutant animals did not uncover additional phenotypes. Taken together, we conclude that there probably exists a high level of redundancy among the GVIA-iPLA$_2$ members in *C. elegans*, and each potentially contributes to phospholipid remodeling during growth and development.

**IPLA-1 Forms a Stable Homodimer in Solution**—Previous work suggests that members of the GVIA subfamily of iPLA$_2$ enzymes assemble as homotetrameric complexes in solution (8, 35). This conclusion is largely based on size exclusion chromatography, which can be influenced significantly by the shape of a protein, and radiation inactivation studies, which are highly dependent on radiation dose (28, 36). To define the oligomeric state of a GVIA-iPLA$_2$ enzyme, we expressed and purified a recombinant form of *C. elegans* IPLA-1, the closest homolog of the human iPLA2β isoforms. A GST tag was initially appended onto the amino terminus of IPLA-1 to enable isolation on glutathione-agarose resin. However, following purification, the GST tag was removed, and all studies were conducted with untagged IPLA-1.

Our results from gel filtration analysis of purified IPLA-1 were consistent with previous work demonstrating that the human and hamster GVIA-iPLA$_2$ enzymes size between the globular standards catalase and thyroglobulin (~300–400 kDa) (8, 35). Specifically, we found that IPLA-1 exhibited a Stokes radius of 6.4 nm by comparing its elution profile with those of characterized standards (Fig. 2A). To determine whether the shape of IPLA-1 may influence its mobility through the size exclusion column, we also measured the sedimentation value of the protein using a glycerol density gradient centrifugation technique. With this approach, we found that IPLA-1 exhibits a sedimentation value of 6.7 S, considerably lower than that expected of a globular homotetrameric complex (Fig. 2B). Using the Svedberg equation (28) (see “Experimental Procedures”), we combined the sedimentation value and Stokes radius of IPLA-1 to estimate its native molecular mass to be ~180 kDa. Given that the predicted molecular mass of IPLA-1, based on its amino acid composition, is ~87 kDa, our hydrodynamic studies indicate that IPLA-1 forms a stable homodimer in solution, with a non-globular conformation.

To determine whether our findings using recombinant IPLA-1 reflect the properties of the endogenous protein, we generated affinity-purified antibodies to characterize native
IPLA-1. To determine the specificity of our antibodies, we first conducted an immunoblot analysis of extracts generated from control and ipl-1 mutant animals, following their separation by SDS-PAGE. Although the antibody exhibited some nonspecific cross-reactivity, we identified a single protein in the control extract, which exhibited a mobility similar to that of recombinant IPLA-1 and was absent in the ipl-1 mutant extract (Fig. 3A). Furthermore, immunoprecipitations from C. elegans embryo extracts conducted using anti-IPLA-1 antibodies recovered endogenous IPLA-1, as determined by mass spectrometry analysis (data not shown). Together, these data validate the functionality and specificity of our antibodies. We therefore used them to examine a C. elegans embryo extract that was fractionated over a gel filtration column and subsequently separated by SDS-PAGE. Our immunoblot analysis indicated that native IPLA-1 exhibits a Stokes radius of 6.2 nm, similar to that observed for recombinant IPLA-1 (Fig. 3B). These data suggest that endogenous IPLA-1 assembles into a complex with a stoichiometry equivalent to that of the recombinant form. Furthermore, based on sequence conservation and hydrodynamic characteristics similar to those of mammalian iPLA2β isoforms, we predict that other members of the GVIA subfamily of iPLA2 enzymes form stable homodimers in solution.

**IPLA-1 Can Also Assemble into a Larger Homotetrameric Complex**—Although our data indicate that IPLA-1 forms a stable homodimeric complex, which remains associated during sedimentation velocity centrifugation (~8 h at 50,000 rpm), higher order oligomeric complexes that are more labile may disassemble under these conditions. To further investigate the possibility that IPLA-1 can generate larger molecular weight complexes in solution, we used MALS. Because the amount of light scattered by a macromolecule is directly proportional to the product of the molecular weight and the protein concentration, we were able to determine the absolute molecular mass of recombinant IPLA-1 immediately following its purification. For these studies, concentrated IPLA-1 was applied onto a high resolution size exclusion column, which was coupled to a MALS detector (SEC-MALS). Analysis of the data using ASTRA software (see “Experimental Procedures”) demonstrated that IPLA-1 assembles into at least two distinct monodisperse complexes, with experimental masses of 350 and 190 kDa (on average), which correspond to homotetrameric and homodimeric complexes, respectively (Fig. 4, A and B). These data strongly suggest that IPLA-1 dimers can self-associate. However, the heterotetrameric complexes of IPLA-1 appear to be less stable and tend to disassemble over time. These data highlight oligomerization as a potentially important point of IPLA2 regulation.

**IPLA-1 Activity Is Stimulated by the Presence of ATP, ADP, and Acidic Phospholipids**—Additional regulatory mechanisms that govern GVIA-iPLA2 activity are largely uncharacterized. To study iPLA2 enzymatic activity, we measured the release of radiolabeled fatty acids from mixed micelles composed of DPPC in the presence of IPLA-1. Similar to previous results examining the activity of human GVIA-2 (2), we found that IPLA-1 exhibits a specific activity of 847 ± 62 nmol/min/mg (Fig. 5A). This level of enzymatic function was independent of calcium and was dramatically attenuated by BEL or MAFP, two
characterized PLA₂ inhibitors (Fig. 5A). Together, these data confirm that IPLA-1 is a bona fide, functional member of the GVIA-iPLA₂ subfamily. Additionally, we found that the presence of ATP was necessary for full activity of IPLA-1 in vitro, again highlighting its similar characteristics to human GVIA-2 (Fig. 5A). The effect of ATP became saturated at a concentration of 1 mM. We further assayed the effect of adding ADP or a non-hydrolyzable analog of ATP (AMP-PNP) to IPLA-1 and found that either was sufficient to stimulate its activity (Fig. 5A). Additionally, the presence or absence of Mg²⁺ did not influence the stimulatory effect of ATP (data not shown). These findings indicate that ATP hydrolysis is not required to promote IPLA-1 lipase activity in vitro. Instead, our data suggest that nucleotide association with the enzyme is sufficient to modulate its function.

To determine whether ATP may affect the oligomeric state of IPLA-1 to promote its elevated activity, we measured the native molecular weight of recombinant IPLA-1 in the presence of ATP. Our findings indicated that 1 mM ATP (with or without Mg²⁺) did not significantly alter the stable homodimeric nature of IPLA-1 (Fig. 5, B and C). In contrast, using SEC-MALS, we found that the presence of ATP increased the experimental masses of the IPLA-1 complexes to 430 and 250 kDa (on average), which correspond to homopentameric and homotrimeric complexes, respectively (Fig. 6A and B). Similar to the role of ATP in stimulating IPLA-1 activity, this effect on oligomerization occurred in the presence or absence of Mg²⁺ (supplemental Fig. S2). Furthermore, a similar change in IPLA-1 complex formation was observed upon the addition of ADP (supplemental Fig. S3). Thus, our data strongly suggest that ATP and ADP enhance lipase activity by promoting the self-association of IPLA-1 into larger molecular weight complexes. Surprisingly, the Stokes radius of IPLA-1 did not considerably differ in the presence or absence of nucleotide, suggesting that the formation of larger oligomers does not significantly affect the architecture of IPLA-1 complexes in solution.

Isoforms of iPLA₂ are known to interact with a number of subcellular organelles, each of which exhibits a unique lipid composition (22–26). To determine whether IPLA-1 activity may be regulated by its exposure to different lipid headgroups, we supplemented the mixed micelle assay with phospholipids commonly found in most cellular membranes, including PS and
**Regulatory Role of Acidic Lipids in iPLA₂ Function**

**IPLA-1 Associates Directly with Multiple Acidic Phospholipids**—One mechanism by which negatively charged phospholipids may enhance IPLA-1 activity is through direct binding and recruitment to micelles that contain the radiolabeled DPPC. To determine whether IPLA-1 associates with acidic phospholipids, we conducted a series of co-flotation experiments, in which liposomes of varying lipid content are incubated with purified IPLA-1 and subsequently floated though an Accudenz gradient. The percentage of IPLA-1 recovered at the top of the gradient is directly related to its affinity for the membrane. Experiments were conducted in the presence of MAFP or BEL to inhibit IPLA-1 lipase activity and to preserve the integrity of the liposomes. Using membranes containing 85% PC and 15% PE, we found that only a small percentage of IPLA-1 was able to bind (less than 5% of total; Fig. 8, A and B). However, as we increased the concentration of PS in the liposomes, the amount of IPLA-1 recovered became similarly elevated. In the presence of membranes with high concentrations of PS, a majority of the total IPLA-1 in the reaction bound to liposomes (Fig. 8A). Furthermore, we found that elevated levels of PG in the membrane also improved binding of IPLA-1 to liposomes. Approximately 40% of the total IPLA-1 added to the reaction bound to liposomes composed of 30% PG, 15% PE, and 55% PC (Fig. 8B). By conducting direct comparisons, we found little difference in the amount of IPLA-1 recovered in co-flotations using liposomes containing equivalent amounts of either PG or PS (Fig. 8C). Notably, the presence of ATP and/or Mg²⁺ did not affect IPLA-1 association with liposomes containing PS (Fig. 8D). We further tested the effects of supplementing liposomes with equivalent mol percentages of PI, CL, PA, PI4P, and PI4,5P₂ on IPLA-1 membrane association. With the exception of PI, which did not promote IPLA-1 membrane binding, we found that the amount of enzyme that co-floated with liposomes was proportional to the overall charge present in the membrane (Fig. 8E). Thus, IPLA-1 bound most avidly to liposomes containing PI4,5P₂, which exhibits the highest negative charge of all lipids tested. However, when we used liposomes that harbored equivalent charge (30% PS versus 10% PI4,5P₂), there was only a minor difference (less than 2-fold) in IPLA-1 binding (Fig. 8F). These data indicate that IPLA-1 exhibits an enhanced affinity for multiple acidic phospholipids, which may play an important role in regulating the distribution and/or function of GVIA-iPLA₂ enzymes in vivo.

To examine the thermodynamics of IPLA-1/membrane interactions, we used isothermal titration calorimetry. This approach takes advantage of the fact that heat is released or absorbed when a protein and a ligand interact. The calorimeter can measure these changes in heat, which are related to the strength of an association (37). We focused our experiments on liposomes with or without PS because this lipid stimulated IPLA-1 activity potently. Titrations using liposomes lacking PS produced heat signals that were small (similar to heats of dilution) and failed to saturate, suggesting that IPLA-1 binds poorly to uncharged membranes (Fig. 9A). In contrast, when liposomes containing PS were titrated into a solution of IPLA-1, we observed relatively large, exothermic heat signals that saturated over the course of the experiment (Fig. 9B). These findings are consistent with data from our co-flotation assays and indicate
that IPLA-1 binds preferentially to membranes harboring the acidic phospholipid PS.

**DISCUSSION**

The highly regulated processes of cell growth, proliferation, and differentiation are accompanied by dramatic changes in membrane architecture, which require the action of numerous lipid-modifying enzymes. In particular, the PLA₂ superfamily of lipases contributes significantly to organelle remodeling and further mediates the production of lipid second messengers that can alter cell signaling during development (2). The 16 known groups of PLA₂ enzymes can be sorted into six main categories: secreted isoforms, calcium-dependent cytosolic isoforms, calcium-independent cytosolic isoforms, platelet-activating factor acetylhydrolase phospholipases, lysosomal phospholipases, and adipose-specific phospholipases. Of these, only the cytosolic isoforms are likely to function in general phospholipid homeostasis and the regulation of organelle morphology (38). However, mechanisms by which these enzymes are regulated and targeted to specific subcellular membranes have remained mostly unknown. Based on amino acid sequence, we identified a set of putative iPLA₂ isoforms in *C. elegans* that exhibit significant homology to the GVIA-iPLA₂ lipases that function in a calcium-independent fashion. Our biochemical characterization of one member of this group indicates that it forms homodimers that can further oligomerize in solution and be stimulated by the presence of acidic phospholipids. Moreover, our data suggest that GVIA-iPLA₂ enzymes bind directly to several acidic phospholipids, which probably plays a key role in regulating their recruitment and distribution in cells.
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acid, a precursor for eicosanoid biosynthesis, which mediates inflammatory and immune responses (39). More recently, it has become clear that PLA2 activity also plays a key role in regulating organelle structure and function. GIVA-cPLA2 has been shown to generate membrane tubules in the Golgi apparatus to foster intra-Golgi transport (40, 41). Similarly, GIVA-iPLA2 plays a role in regulating the morphology of the ER-Golgi intermediate compartment, a sorting hub for anterograde cargoes destined for the Golgi and retrograde cargoes directed to the ER (26). GIVA-iPLA2, additionally functions at mitochondria during apoptosis and has been implicated in rupturing the outer mitochondrial membrane to release cytochrome c into the cytoplasm (42, 43). Mice harboring a deletion in iPLA2β, the gene encoding GIVA-iPLA2, exhibit premature axonal degeneration, further indicating that the function of the enzyme is not limited to a unique subcellular site (21). These findings raise an important question. How is GIVA-iPLA2 recruited onto multiple, distinct organelles? Our study offers some insight into this issue. We speculate that acidic phospholipids, which are found in all intracellular membranes, play a key role in regulating GIVA-iPLA2 distribution and activity. In contrast to GIVA-cPLA2, which is stimulated potently by PI4,5P2 specifically (44), we found that GIVA-iPLA2 is activated by numerous acidic phospholipids in a manner that is dependent on overall charge as opposed to headgroup specificity. These findings highlight a new distinction between these two groups of phospholipase A2 enzymes. Other factors must provide additional specificity in targeting the GIVA-iPLA2 lipases. In particular, protein/protein interactions on various organelles probably foster GIVA-iPLA2 recruitment and/or regulation. At the ER-Golgi intermediate compartment, GIVA-iPLA2 binds to specific Arf-type GTases (Arf1 and Arf4), which precisely control its function in membrane tubulation (26). A requirement for coincidence detection of both a protein and acidic phospholipids may provide sufficient stringency to prevent constitutive lipase activity while still enabling rapid changes in GIVA-iPLA2 localization to control the architecture of multiple organelles. Notably, deuterium exchange experiments were recently conducted using human GIVA-2 to monitor its association with neutral phospholipid membranes (45). It will be interesting to determine how the presence of acidic phospholipids influences GIVA-2 membrane binding and to dissect the regions of GIVA-2 that respond specifically to charged and uncharged lipid headgroups.

Oligomerization of GIVA-iPLA2 Isoforms and the Role of Nucleotides—Size exclusion chromatography studies indicate that human GIVA-2 and C. elegans IPLA-1 exhibit comparable elution profiles, suggesting that they assemble into complexes of similar stoichiometries. Although previous work raised the possibility that GIVA-2 forms a homotetramer (8, 35), it remained unclear how such a complex might form. For the first time, our analysis of recombinant IPLA-1 demonstrates that it forms a homodimer in solution, which can self-associate to generate a homotetrameric complex. Surprisingly, the IPLA-1 homodimer exhibits a Stokes radius nearly identical to that of the tetrameric form, suggesting that they both adopt similar conformations although their molecular weights differ significantly. Crystallography studies of other proteins harboring tandem ankyrin repeat domains have revealed that they exhibit highly extended configurations. In Bcl-3, a member of the IκB protein family, its seven ankyrin repeats form an elongated structure with approximate dimensions of 75 × 25 × 25 Å (46). Notably, IPLA-1 and GIVA-2 both harbor seven putative ankyrin repeats, suggesting that their amino termini adopt a conformation similar to Bcl-3. Together with the high level of sequence similarity observed among IPLA-1 and other GIVA-iPLA2 isoforms, we speculate that all members of this group initially form elongated homodimers that can further self-associate to generate larger molecular weight complexes. Such an assembly pathway may be under tight control in vivo, providing an additional mechanism to regulate iPLA2 activity.

In addition to the patatin-like lipase domain, several members of the GIVA-iPLA2 subfamily contain a conserved glycinerich motif, predicted to associate with ATP (47). Both human GIVA-2 (8) and C. elegans IPLA-1 are stimulated by the presence of ATP and ADP in vitro, prompting us to consider whether the addition of nucleotides may promote the formation of larger molecular weight complexes. Indeed, our studies demonstrated that 1 mM ATP or 1 mM ADP increases the subunit stoichiometry of IPLA-1 complexes, although the larger homo-oligomers remain labile and appear to disassemble during the course of glycerol gradient sedimentation experiments, which require 8 h of centrifugation time. Previous work suggested that ATP may not directly activate iPLA2 isoforms but instead may limit its denaturation during an enzymatic assay (48). Our data are consistent with this idea but further argue that ATP and ADP promote iPLA2 self-association. Thus, GIVA-iPLA2 function in vivo may be sensitive to the metabolic state of the cell, and fluctuations in local ADP/ATP levels may substantially influence lipase activity.

Phospholipase A2 Function in C. elegans—Mammalian cells express a single gene encoding multiple alternatively spliced GIVA-iPLA2 isoforms. In contrast, the C. elegans genome possesses seven unique genes that generate putative members of this group. It is unclear why this subset of PLA2 enzymes has undergone such a dramatic expansion in worms. However, it is notable that we were unable to identify any calcium-dependent PLA2 enzymes in C. elegans, based on sequence homology. This finding suggests that PLA2-dependent organelle remodeling and membrane homeostasis relies mostly on the expanded set of GIVA-iPLA2 enzymes. Our genetic analysis of mutant animals harboring deletion mutations within several of the GIVA-iPLA2 genes suggests that there exists a high level of redundancy among these enzymes. However, we were able to score significant defects in reproduction rate in animals lacking multiple GIVA-iPLA2 isoforms, suggesting a critical role for this group of phospholipases during oogenesis, ovulation, and/or embryogenesis. Previous work indicates that the GVII-PLA2 enzymes, referred to as the platelet-activating factor acetylhydrolase phospholipases PAF-1 and PAF-2, fulfill a separate essential function in epithelial morphogenesis in C. elegans (34). Additionally, based on amino acid sequence, at least two secretory PLA2 isoforms are expressed in worms, encoded by the open reading frames C07E3.9 and Y69A2AL.2. However, it remains unclear whether these putative enzymes are essential for normal development. Nonetheless, the overall limited num-
number of PLA2 isoforms in C. elegans makes it an attractive system to study the essential properties of these enzymes. In the future, it will be interesting to define the individual contributions of various PLA2 isoforms and uncover the precise importance of deacylation of phospholipids at the sn2 position during different stages of development.

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